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Incorporation of [1,5 - $^{14}C_2$] Citrate into the Various Fatty Acids of the Mouse Liver

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The works of several groups of researchers have enabled the attribution to citrate of three essential roles in the synthesis of fatty acids: that of precursor[1], that of activator and that of carrier of the acetyl group. The incorporation into the fatty acids of the acetyl group resulting from the breakdown of citric acid is even greater than that of the acetate[2-5]; its activator[6-8] role results from its effect on acetyl CoA carboxylase[9,10]. Finally, citric acid enables acetyl CoA to leave the mitochondria[10-12]; and it is outside of these cells that the enzymes for the synthesis of fatty acids seem to be the most active[13,28]. However, the role of citrate in the synthesis of fatty acids in living animals has not yet been precisely described. Its role as an activator seems debatable *in vivo* because the concentrations of citrate necessary to cause an activation of the synthesis of fatty acids *in vitro* greatly exceeds the quantities present in the tissues[6-8,14].

In this work, we are especially interested in the role of citrate as the precursor in the synthesis of fatty acids in the mouse. We thus anticipate obtaining information as well on its role as a carrier because the two functions are closely related. It could not really be said that citrate carries the largest fraction of the acetyl CoA from the mitochondria if it was not a good precursor. We have also studied its incorporation into the principal fatty acids of the liver and carcass and also in the different cellular parts of the liver, so as to acquire information on the localization of lipogenesis. To be able to validly discuss the problem of its dilution by endogenous citrate, we were also interested in its incorporation into fatty acids and its penetration into the liver and adipose tissue as a function of time. A complementary study on the incorporation of (4-^{14}C)-aspartate into fatty acids enabled the additional suggestion of a particular mechanism for the synthesis of stearic acid from citrate.

Material and Methods

Preparation and Treatment of Animals

The mice received ad libitum "Nafag" cubes (compressed food for mice) on the days preceding the experiment. A last meal comprised of 1 g of food succeeding a 3-hour fast was ingested 1 hour before the administration of radioactive precursors.

Experiments 1 and 2 - Some mice received 10 µC (Exp. 2: 50 µC) of [1-5-$^{14}C_2$]-citrate (15.4 µC/mmole)and 10 µC (Exp. 2: 50 µC) of [2-^{3}H] acetate (500 µC/mmole) and were sacrificed 30 minutes afterwards. The organs were immediately removed and fixed by alcoholic potash before extracting the fatty acids from them (Exp. 1) or treated to separate the different cellular fractions (Exp. 2).

Experiment 3 - Mice received an injection of 100 µC of [4-^{14}C] aspartate (20 µC/mmole) and 10 µC of [2-^{3}H] acetate (500 µC/mmole) and were sacrificed 12 minutes later. The fatty acids from the carcass of two mice were extracted, isolated by chromatography and the radioactivity of palmitic, stearic and oleic acids was measured.

Experiment 4 - Three groups of 5 mice received in intravenous injection 12 µC of [1,5$^{-14}C_2$] citrate and 15 µC of [2-^{3}H] acetate and were sacrificed 12, 30, 60 and 120 minutes afterward. Bits of tissue were quickly sampled to determine from them the gross activity. The rest of the animal was fixed with alcoholic potash and the fatty acids extracted.

Preparation of Cellular Liver Fractions

The livers (Exp. 2) were chilled in ice, weighted, then homogenized between 0° and 4° in 10 vol. of 0.25 M saccharose using a Potter equipped with a Teflon piston. The cellular fractions were separated according to the technique of Christ and Hulsmann[17]. The homogenate was centrifuged at 800 x g for 3 minutes to eliminate the nuclei and cellular debris; the supernatant was centrifuged at 4500 x g for 10 minutes to isolate the mitochondria; the supernatant of this second centrifugation was centrifuged for 30 minutes at 100,000 x g to obtain the real supernatant and the microsomes. The purity of the fractions was verified by an electron microscope thanks to the willingness of the Institute of Histology and Embryology of the University of Geneva. An aliquot of each

fraction, suspended in 0.25 M saccharose was used to measure the proteins by the biuret method.[18]

Saponification, Extraction and Separation of the Fatty Acids

The different tissues and cellular fractions of liver were saponified using alcohol KOH, then the fatty acids were extracted with petroleum ether after the elimination of the unsaponifiable fraction. Methyl esters were prepared and extracted according to the method of Lasker and Theilaker[15] (Exps. 1 and 3). Palmitic, stearic and oleic acids were separated by thin-layer chromatography[16] and by gas-liquid chromatography (column: apiezon L 15% on Aeropak, length 10 feet, diameter 3/8 inch; temperature 200°; gas vector: helium; yield: 80 ml/min), the methyl esters were hydrolyzed and the fatty acids extracted so as to avoid all eventual contamination by the stable phase. The radioactivity of the fatty acids was measured by liquid scintillation (15 ml of a solution containing 5 g of diphenyloxazole and 300 mg of 1,4-bis-(5-phenyloxazolyl-2) benzene per liter of toluene. "Quenching" was estimated according to Hendler[19].

Determination of the Gross Activity of the Tissues (Exp. 4)

50 to 100 mg of liver, adipose tissue or muscle (weight of fresh tissue) were removed, weighed and placed in a flask containing 1 ml of Soluene (Packard), then stored in a water-bath at 60° for about 12 hours or until the complete dissolution of the tissue; 14 ml of scintillating liquid of similar composition as that described for the fatty acids, was added to each flask before determining the radioactivity by means of liquid scintillator.

Results

Experiment 1 - Citrate was slightly incorporated into the fatty acids of the carcass and liver, as compared to acetate, more so in those of the kidney and intestine. If the total incorporation is considered, it is found that the fatty acids of the intestine incorporate more citrate than those of the liver. The importance of these two organs is the inverse with acetate which is better incorporated into the liver than citrate.

The $^{3}H/^{14}C$ ratio of palmitic acid is about two times greater than that of stearic acid in the carcass. This is not the case in the liver (Table 1) where oleic acid presents the lowest $^{3}H/^{14}C$ ratio.

Experiment 2 - Thirty minutes after the injection of [1,5-$^{14}C_2$] citrate and [2-^{3}H] acetate into live animals, we found in fatty acids of the supernatant and the hepatic mitochondria, the same proportion of the two precursors. In the mitochondria, [2-^{3}H] acetate was better incorporated than [1,5-$^{14}C_2$] citrate (Table 2).

Experiment 3 - As would be expected [4-^{14}C] aspartate was incorporated very little into the fatty acids in relation to the [2-^{3}H] acetate. Compared to acetate, the [4-^{14}C] aspartate participates more actively in the synthesis of stearic acid and of oleic acid than in that of palmitic acid. For an identical quantity of the two radioactive precursors, the $^{3}H/^{14}C$ ratios of the three fatty acids are 166 and 144 for palmitic acid, 83 and 63 for stearic acid, 50 and 23 for oleic acid.

Experiment 4 - The $^{3}H/^{14}C$ ratios in liver, adipose tissue and muscle observed 12, 30 and 60 minutes after the administration of [2-^{3}H] acetate and [1,5-$^{14}C_2$] citrate increased significantly as a function of time (Table 3).

Table 1. Incorporation of [1,5-$^{14}C_2$] citrate and [2-^{3}H] Acetate into Fatty Acids

Mice received 10 μC of citrate and 10 μC of acetate in intravenous injections and were killed 30 minutes later. The activity was expressed as disintegration/minute per mg of fatty acids (specific activity) or as % of radioactivity of the total fatty acids found in each organ. The standard deviations are indicated in parentheses.

Precursor	Carcass	Liver	Kidney	Intestine
[1,5-$^{14}C_2$] Citrate	25.9 (7.2)	41.7 (14.5)	38.8 (7.5)	373 (49)
[2-^{3}H] Acetate	67.7 (12.5)	273.3 (28.5)	43.0 (5.8)	343 (66)
$^{3}H/^{14}C$	2.6 (0.6)	6.5 (0.6)	1.15(0.08)	0.9 (0.09)
[1,5-$^{14}C_2$] Citrate	54.2%	16.4%	1.2%	28.2%
[2-^{3}H] Acetate	63.9%	27.3%	0.6%	8.2%
$^{3}H/^{14}C$ Palmitic acid	3.4 (0.45)	6.4 (0.81)		
Stearic acid	1.7 (0.27)	7.4 (1.10)		
Oleic acid	3.5 (0.25)	4.3 (0.25)		

Table 2. Incorporation of [1,5-$^{14}C_2$] Citrate and [2-^{3}H] Acetate into Cellular Fractions of Mouse Liver

The animals received in intravenous injection 50 µC of [1,5-$^{14}C_2$] citrate and 50 µC of [2-^{3}H] acetate and were sacrificed after 30 minutes. The radioactivity of the fatty acids found in the three fractions (supernatant, microsomes, mitochondria) was considered to be equal to 100.

	Mouse No.	1	2	3	4	M
Supernatant	^{14}C	23.4	21.3	21.3	31.5	
	^{3}H	18.7	22.4	17.7	31.2	
	$^{3}H/^{14}C$	0.80	1.05	0.83	0.99	0.92
Microsomes	^{14}C	65.3	60.7	41.7	54.8	
	^{3}H	69.4	59.7	44.5	54.0	
	$^{3}H/^{14}C$	1.06	0.98	1.06	0.99	1.02
Mitochondria	^{14}C	11.3	18.0	37.0	13.7	
	^{3}H	11.9	17.9	37.8	14.8	
	$^{3}H/^{14}C$	1.05	1.0	1.02	1.09	1.01

Table 3. Total Specific Activity of the Liver, Adipose Tissue and Muscle After Intravenous Injection of [2-^{3}H] Acetate and [1,5-$^{14}C_2$] Citrate.

The activities are given as disintegration/min per mg of tissue. The significances (Student's test) are indicated in relation to the results obtained after 12 minutes. The $^{3}H/^{14}C$ ratios are the averages of the $^{3}H/^{14}C$ individual values.

Time (min)	Liver ^{14}C	Liver ^{3}H	Liver $^{3}H/^{14}C$	Adipose Tissue ^{14}C	Adipose Tissue ^{3}H	Adipose Tissue $^{3}H/^{14}C$	Muscle ^{14}C	Muscle ^{3}H	Muscle $^{3}H/^{14}C$
12	797	1430	2.0	154	172	1.0	370	1187	3.1
30	413	990	2.3	86	105	1.2	293	945	3.9
60	276	939	3.4	31	112	3.6	183	1161	5.7
P	<0.01		<0.05	<0.02		<0.001	<0.01		<0.001

Discussion

A major fact merits particular consideration: the citrate is incorporated to a much less degree than acetate into the fatty acids of the liver and carcass. Actually the $^{3}H/^{14}C$ ratios of the fatty acids in the organs where lipogenesis most easily takes place, in other words, the liver and the adipose tissue, are around 3.8. Considering the fact that [2-^{3}H] acetate is about 5 times less incorporated than [2-^{14}C] acetate into the fatty acids *in vivo* as well as *in vitro*, the incorporation of citrate into fatty acids would then be about 20 times less than that which could be obtained utilizing [2-^{14}C] acetate. However, since only the radioactivity of C-1 of the citrate could be recovered in the fatty acids[22], this proportion would be brought down to 10.

Before concluding that citrate is really a poor precursor of fatty acids, it is necessary to know whether its slight incorporation is due to the fact that exogenous citrate is the target of significant dilution by endogenous citrate or whether it can diffuse easily across the plasma membrane, or whether it is used for other purposes. We will now consider these various points.

(1) Because of the difficulties encountered by the citrate in penetrating the mitochondria it is highly probable that exogenous radioactive citrate, will liberate its acetyl CoA slowly in the cytoplasm which is precisely where the catalyzing enzyme for its breakdown is found.[14,23,24] This would permit the confirmation of the almost identical behavior of citrate and acetate in the synthesis of the fatty acids of the supernatant, the microsomes and the mitochondria.

The [2-^{3}H] acetate would undoubtedly be directly activated in the cytoplasm[26], in other words, without previous transport by the mitochondria. Thus, the dilution exerted by the endogenous citrate must take place in the same way on the two precursors studied.

(2) A too slow penetration of citrate into the cells does not suffice either to explain its poor incorporation. The measure of gross radioactivity as a function of time (Table 3) shows that after 12 minutes, citrate penetrated as well as acetate into the adipose tissue and in the liver, only two times less. The difference is more significant for muscle but this tissue hardly participates at all in the synthesis of fatty acids. The $^{3}H/^{14}C$ ratio goes up in the three tissues at the same time as the actual activity diminishes. This indicates that the penetration is largely terminated and that the ^{14}C of citrate is subject to

more rapid combustion than acetate, especially in muscle and liver. The major part of the citrate which had first of all penetrated into the adipose tissue, probably does not burn in this tissue.

(3) The low incorporation of citrate could also be explained at first glance in the following way: citrate, after having penetrated into the cytoplasm would escape the effect of the splitting enzyme and thus would be lost for the synthesis of fatty acids. Actually, the first steps of the tricarboxylic cycle which brings about the transformation of citrate into α-ketoglutarate can take in the cytoplasm (refs. 26, 27). The interpretation of the results would not however be modified because if this pathway was competitive, this would still mean that the enzyme splitting the citrate is less active. This hypothesis, however, does not agree with the increase of the radioactivity of the fatty acids as a function of time observed after the administration of citrate (unpublished results). This last observation thus means that since the citrate remains in the cell, the activity of the splitting enzyme in vivo is very weak, as has already been suggested by Foster and Srere[14].

If now we compare the incorporation of citrate to that of acetate into the various fatty acids, we see that in the carcass of live animals citrate is used in preference to acetate for the synthesis of stearic acid. But in the presence of the supernatant of pigeon liver, citrate participates above all in the synthesis of palmitic acid[3]. These differences have made us think that is not the part of citrate which gives rise to acetyl-CoA which is better incorporated into stearic acid, but actually that which corresponds to oxaloacetate. Actually, it is difficult to accept that the two acetates, the one administered as is and the other coming from citrate behave differently. To verify this hypothesis, we injected [4-^{14}C] aspartate which like [1,5-$^{14}C_2$] citrate produced labeled oxaloacetate in position 4. The oxaloacetate could participate in the synthesis of fatty acids by taking the path described by Kallen and Lowenstein[27] (Fig. 4) and according to which oxaloacetate could by oxidative decarboxylation be directly changed into malonyl-CoA. We found only slight incorporation of aspartate into fatty acids. This fact is not surprising since this pathway can only be accessory, this precursor having many other purposes, especially the synthesis of proteins. On the other hand, the proposed pathway necessitates a deacylation, then a corresponding reacylation, reactions which are really of very little

significance. It stands out, however, from these results that in the carcass, the synthesis of stearic acid is favored from aspartate as it is from citrate. This would imply that the direct pathway, oxaloacetate → malonyl-CoA preferentially furnishes the malonyl-CoA necessary to microsomal elongation.

Summary

Mice received [1,5-$^{14}C_2$] citrate, [2-^{3}H] acetate or [4-^{14}C] aspartate even by intravenous injections or by intubation. The radioactivity of the fatty acids of the liver, the carcass, the intestine and the kidney was measured as well as that of the fatty acids of the different cellular fractions of the liver. The total specific activity of the liver, the adipose tissue and the muscle was also measured over time. The [1,5-$^{14}C_2$] citrate was incorporated to a lesser extent than acetate into the fatty acids of the carcass and the liver. In the carcass, citrate was utilized in preference to the acetate for the synthesis of stearic acid; the same phenomenon occurs from [4-^{14}C] aspartate. The [1,5-$^{14}C_2$] citrate and the [2-^{3}H] acetate participate in the same proportions in the synthesis of fatty acids in the microsomes and supernatant; the incorporation of acetate was favored in the mitochondria. The $^{3}H/^{14}C$ ratios of gross activities in the liver, adipose tissue and muscle observed after the administration of [2-^{3}H] acetate and [1,5-$^{14}C_2$) citrate all increased as a function of time. The role of citrate as a precursor in the synthesis of fatty acids is discussed on the basis of these results.

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BIOCHIMICA ET BIOPHYSICA ACTA

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INCORPORATION DU [1,5-$^{14}C_2$]CITRATE DANS LES DIFFÉRENTS ACIDES GRAS DE LA SOURIS VIVANTE

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SUMMARY

[1,5-$^{14}C_2$]Citrate or [4-^{14}C]aspartate together with [2-^{3}H]acetate were applied to mice intravenously or by intubation. The incorporation of these precursors into fatty acids of the liver, carcass, intestine and kidney was studied at different time intervals after the administration. The radioactivity of fatty acids present in liver subcellular fractions was also measured.

[1,5-$^{14}C_2$]Citrate was incorporated into fatty acids of carcass and liver to a much lesser extent than [2-^{3}H]acetate.

Moreover, it was found that in contrast with [2-^{3}H]acetate the radioactivity of [1,5-$^{14}C_2$]citrate and [4-^{14}C]aspartate appeared predominantly in the stearic acid.

The experiments with liver tissue fractions revealed that [1,5-$^{14}C_2$]citrate and [2-^{3}H]acetate were incorporated into fatty acids of microsomes and of supernatant in the same proportion whereas in mitochondrial fraction the acetate incorporation predominated. From the time-course experiments it may be concluded that the $^{3}H/^{14}C$ ratio found in liver, adipose and muscle tissues after the administration of [2-^{3}H]-acetate as well as [1,5-$^{14}C_2$]citrate increased as a function of time.

These results are discussed in relation to the role of citrate in the regulation of fatty acid synthesis.

INTRODUCTION

Les travaux de plusieurs groupes de chercheurs ont permis d'attribuer au citrate trois rôles essentiels dans la synthèse des acides gras: celui de précurseur[1], celui d'activateur et celui de transporteur du groupe acétyle. L'incorporation dans les acides gras du groupe acétyle résultant du clivage de l'acide citrique serait même plus forte que celle de l'acétate[2-5]; son rôle d'activateur[6-8] résulterait d'une action sur l'acétyl-CoA carboxylase[9,10]. Enfin l'acide citrique permettrait la sortie de l'acétyl-CoA hors des mitochondries[10-12]; or, c'est à l'extérieur de ces particules que les enzymes de la synthèse des acides gras paraissent les plus actifs[13,28]. Cependant, le rôle du citrate dans la synthèse des acides gras n'avait pas encore été précisé chez l'animal vivant.

Son rôle d'activateur semble discutable *in vivo* car les concentrations en citrate nécessaires pour provoquer une activation de la synthèse des acides gras *in vitro*, excèdent de beaucoup les quantités présentes dans les tissus[6-8,14].

Dans ce travail, nous nous sommes surtout intéressés au rôle du citrate comme précurseur de la synthèse des acides gras chez la souris vivante. Nous escomptions ainsi obtenir également des renseignements sur son rôle de transporteur, car ces deux fonctions sont étroitement liées. On ne peut admettre en effet, que le citrate transporte la plus grande partie de l'acétyl-CoA hors des mitochondries, s'il n'est lui-même un bon précurseur. Nous avons d'autre part étudié son incorporation dans les principaux acides gras du foie et de la carcasse et aussi dans les différentes fractions cellulaires du foie, afin d'acquérir des informations sur la localisation de la lipogenèse. Pour pouvoir discuter plus valablement le problème de sa dilution par le citrate endogène, nous nous sommes également intéressés à son incorporation dans les acides gras et à sa pénétration dans le foie et le tissu adipeux en fonction du temps. Une étude complémentaire de l'incorporation du [4-^{14}C]-aspartate dans les acides gras a permis en outre de suggérer l'existence d'un mécanisme particulier permettant la synthèse de l'acide stéarique à partir du citrate.

MATÉRIEL ET MÉTHODES

Préparation et traitement des animaux

Des souris reçoivent *ad libitum* des cubes "Nafag" (aliment comprimé pour souris) pendant les jours précédant l'expérience. Un dernier repas constitué de 1 g de nourriture succédant à un jeûne de 3 h est ingéré 1 h avant l'administration des précurseurs radioactifs.

Expériences 1 et 2. Des souris reçoivent per os 10 μC (Exp. 2: 50 μC) de [1,5-$^{14}C_2$]-citrate (15.4 μC/mmole) et 10 μC (Exp. 2: 50 μC) de [2-^{3}H] acétate (500 μC/mmole) et sont sacrifiées 30 min après. Les organes sont prélevés immédiatement et fixés au moyen de potasse alcoolique afin d'en extraire les acides gras (Exp. 1) ou traités pour en séparer d'abord les différentes fractions cellulaires (Exp. 2).

Expérience 3. Des souris reçoivent une injection de 100 μC de [4-^{14}C]aspartate (20 μC/mmole) et de 10 μC de [2-^{3}H]acétate (500 μC/mmole) et sont sacrifiées 12 min après. Les acides gras de la carcasse de deux souris sont extraits, isolés par chromatographie et la radioactivité des acides palmitique, stéarique et oléique est mesurée.

Expérience 4. Trois groupes de cinq souris reçoivent en injection intraveineuse 12 μC de [1,5-$^{14}C_2$]citrate et 15 μC de [2-^{3}H]acétate et sont sacrifiées 12, 30, 60 et 120 min après. Des fragments de tissus sont aussitôt prélevés pour en déterminer l'activité brute. Le reste de l'animal est fixé par de la potasse alcoolique et les acides gras sont extraits.

Préparation des fractions cellulaires du foie

Les foies (Exp. 2) sont refroidis dans la glace, pesés puis homogénéisés entre 0° et 4° dans 10 vol. de saccharose 0.25 M au moyen d'un Potter muni d'un piston en Teflon. Les fractions cellulaires sont séparées selon la technique de CHRIST ET HÜLSMANN[17]. L'homogénat est centrifugé à 800 × *g* pendant 3 min pour éliminer les noyaux et les débris cellulaires; le surnageant est centrifugé à 4500 × *g* pendant 10 min pour en isoler les mitochondries; le surnageant de cette seconde centrifugation est

centrifugé pendant 30 min à 100000 × *g* pour obtenir le surnageant proprement dit et les microsomes. La pureté des fractions a été contrôlée au microscope électronique grâce à l'obligeance de l'Institut d'Histologie et d'Embryologie de l'Université de Genève. Une partie aliquote de chaque fraction, suspendue dans du saccharose 0.25 M, est utilisée pour doser les protéines par la méthode du biuret[18].

Saponification, extraction et séparation des acides gras

Les différents tissus et les fractions cellulaires du foie sont saponifiés au moyen de KOH alcoolique, puis les acides gras extraits à l'éther de pétrole, après élimination de l'insaponifiable. Les esters méthyliques sont préparés et extraits selon la méthode de LASKER ET THEILAKER[17] (Exps. 1 et 3). Les acides palmitique, stéarique et oléique sont séparés par chromatographie en couche mince[16] et par chromatographie gaz-liquide préparative (colonne: apiezon L 15% sur Aeropak, longueur 10 pieds, diamètre 3/8 pouce; température 200°; gaz vecteur: hélium; débit: 80 ml/min). Les esters méthyliques sont hydrolysés et les acides gras extraits pour éviter toute contamination éventuelle par la phase stationnaire. La radioactivité des acides gras est mesurée par scintillation liquide (15 ml d'une solution contenant 5 g de diphényloxazole et 300 mg de 1,4-bis-(5-phényloxazolyl-2)benzène par l de toluène). Le "quenching" est estimé selon HENDLER[19].

Détermination des activités brutes des tissus (Exp. 4)

50 à 100 mg de foie, de tissu adipeux ou de muscle (poids de tissu frais) sont prélevés, pesés et placés dans un flacon contenant 1 ml de Soluène (Packard), puis entreposés dans un bain-marie à 60° pendant environ 12 h, soit jusqu'à dissolution complète du tissu; 14 ml de mélange scintillant de composition analogue à celle décrite pour les acides gras sont ajoutés à chaque flacon avant de déterminer la radioactivité au moyen du scintillateur liquide.

RÉSULTATS

Expérience 1. Le citrate est peu incorporé comparativement à l'acétate dans les acides gras de la carcasse et du foie, mieux dans ceux du rein et de l'intestin. Si l'on considère les incorporations totales, on constate que les acides gras de l'intestin incorporent plus de citrate que ceux du foie. L'importance de ces deux organes s'inverse avec l'acétate qui dans le foie s'incorpore mieux que le citrate.

Le rapport $^{3}H/^{14}C$ de l'acide palmitique est environ 2 fois plus grand que celui de l'acide stéarique dans la carcasse. Ce n'est pas le cas dans le foie (Tableau I) où c'est l'acide oléique qui présente le rapport $^{3}H/^{14}C$ le plus petit.

Expérience 2. 30 min après injection de [1,5-$^{14}C_2$]citrate et de [2-^{3}H]acétate à des animaux vivants, on retrouve dans les acides gras du surnageant et des microsomes hépatiques la même proportion des deux précurseurs. Dans les mitochondries le [2-^{3}H]acétate est mieux incorporé que le [1,5-$^{14}C_2$]citrate (Tableau II).

Expérience 3. Comme on pouvait s'y attendre, le [4-^{14}C]aspartate s'incorpore très faiblement dans les acides gras par rapport au [2-^{3}H]acétate. Comparativement à l'acétate, le [4-^{14}C]aspartate participe plus intensément à la synthèse de l'acide stéarique et à celle de l'oléique qu'à celle du palmitique. Pour une quantité identique des deux précurseurs radioactifs les rapports $^{3}H/^{14}C$ des trois acides gras sont 166 et

TABLEAU I

INCORPORATION DU [1,5-$^{14}C_2$]CITRATE ET DU [2-^{3}H]ACÉTATE DANS LES ACIDES GRAS

Les souris reçoivent 10 μC de citrate et 10 μC d'acétate en injection intraveineuse et sont exécutées 30 min plus tard. Les activités sont exprimées en désint./min par mg d'acides gras (activités spécifiques) ou en % de la radioactivité des acides gras totaux retrouvés dans chaque organe. Les écarts-types sont indiqués entre parenthèses.

Précurseur	*Carcasse*	*Foie*	*Rein*	*Intestin*
[1,5-$^{14}C_2$]Citrate	25.9 (7.2)	41.7 (14.5)	38.8 (7.5)	373 (49)
[2-^{3}H]Acétate	67.7 (12.5)	273.3 (28.5)	43.0 (5.8)	343 (66)
^{3}H/^{14}C	2.6 (0.6)	6.5 (0.6)	1.15 (0.08)	0.9 (0.09)
[1,5-$^{14}C_2$]Citrate	54.2%	16.4%	1.2%	28.2%
[2-^{3}H]Acétate	63.9%	27.3%	0.6%	8.2%
^{3}H/^{14}C acide palmitique	3.4 (0.45)	6.4 (0.81)		
acide stéarique	1.7 (0.27)	7.4 (1.10)		
acide oléique	3.5 (0.25)	4.3 (0.25)		

TABLEAU II

INCORPORATION DU [1,5-$^{14}C_2$]CITRATE ET DU [2-^{3}H]ACÉTATE DANS LES FRACTIONS CELLULAIRES DU FOIE DE SOURIS

Les animaux reçoivent en injection intraveineuse 50 μC de [1,5-$^{14}C_2$]citrate et 50 μC de [2-^{3}H]-acétate et sont sacrifiés après 30 min. La radioactivité des acides gras retrouvés dans les trois fractions (surnageant, microsomes, mitochondries) est considérée comme égale à 100.

	Souris No.	*1*	*2*	*3*	*4*	*M*
Surnageant	^{14}C	23.4	21.3	21.3	31.5	
	^{3}H	18.7	22.4	17.7	31.2	
	^{3}H/^{14}C	0.80	1.05	0.83	0.99	0.92
Microsomes	^{14}C	65.3	60.7	41.7	54.8	
	^{3}H	69.4	59.7	44.5	54.0	
	^{3}H/^{14}C	1.06	0.98	1.06	0.99	1.02
Mitochondries	^{14}C	11.3	18.0	37.0	13.7	
	^{3}H	11.9	17.9	37.8	14.8	
	^{3}H/^{14}C	1.05	1.0	1.02	1.09	1.01

144 pour l'acide palmitique, 83 et 63 pour l'acide stéarique, 50 et 23 pour l'acide oléique.

Expérience 4. Les rapports ^{3}H/^{14}C des activités brutes du foie, du tissu adipeux et du muscle observés 12, 30 et 60 min après administration de [2-^{3}H]acétate et de [1,5-$^{14}C_2$]citrate croissent tous significativement en fonction du temps (Tableau III).

TABLEAU III

ACTIVITÉ SPÉCIFIQUE TOTALE DU FOIE, DU TISSU ADIPEUX ET DU MUSCLE APRÈS INJECTION INTRAVEINEUSE DE [2-^{3}H]ACÉTATE ET DE [1,5-$^{14}C_2$]CITRATE

Les activités sont données en désint./min par mg de tissu. Les significations (test de Student) sont indiquées par rapport aux résultats obtenus après 12 min. Les rapports ^{3}H/^{14}C sont les moyennes des ^{3}H/^{14}C individuels.

Temps	*Foie*			*Tissu adipeux*			*Muscle*		
(min)	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C
12	797	1430	2.0	154	172	1.0	370	1187	3.1
30	413	990	2.3	86	105	1.2	293	945	3.9
60	276	939	3.4	31	112	3.6	183	1161	5.7
P	<0.01		<0.05	<0.02		<0.001	<0.01		<0.001

DISCUSSION

Un premier fait mérite particulièrement d'être pris en considération: le citrate est beaucoup plus faiblement incorporé que l'acétate dans les acides gras du foie et de la carcasse. En effet, les rapports $^{3}H/^{14}C$ des acides gras des organes où s'effectue le plus aisément la lipogenèse, c'est-à-dire le foie et le tissu adipeux, sont voisins de 3.8. En tenant compte du fait que le [2-^{3}H]acétate est environ 5 fois moins incorporé que le [2-^{14}C]acétate dans les acides gras aussi bien *in vivo*[20] qu'*in vitro*[21], l'incorporation du citrate dans les acides gras serait donc environ 20 fois plus faible que celle que l'on aurait obtenue en utilisant du [2-^{14}C]acétate. Toutefois, puisque seule la radioactivité du C-1 du citrate peut se retrouver dans les acides gras[22], cette proportion serait ramenée à 10.

Avant de conclure que le citrate est réellement un mauvais précurseur des acides gras, il importe de savoir si sa faible incorporation tient au fait que le citrate exogène est l'objet d'une importante dilution par le citrate endogène, ou qu'il ne peut diffuser facilement à travers la membrane plasmatique, ou encore qu'il est utilisé à d'autres fins. Nous allons maintenant considérer ces divers points.

(1) En raison des difficultés rencontrées par le citrate pour pénétrer dans les mitochondries, il est fort probable que le citrate radioactif exogène, libérera lentement son acétyl-CoA dans le cytoplasme où se trouve précisément l'enzyme catalysant son clivage[14,23,24]. C'est ce que permet d'affirmer le comportement presque identique du citrate et de l'acétate dans la synthèse des acides gras du surnageant, des microsomes et des mitochondries.

Le [2-^{3}H]acétate sera sans doute activé directement dans le cytoplasme[25], c'est-à-dire sans passage préalable par les mitochondries. Ainsi, la dilution exercée par le citrate endogène devrait s'effectuer de la même façon sur les deux précurseurs étudiés.

(2) Une trop lente pénétration du citrate dans les cellules ne suffit pas non plus à expliquer sa mauvaise incorporation. La mesure des radioactivités brutes en fonction du temps (Tableau III) montre qu'après 12 min, le citrate a pénétré aussi bien que l'acétate dans le tissu adipeux, et dans le foie seulement 2 fois moins bien. La différence est plus importante pour le muscle, mais ce tissu ne participe guère à la synthèse des acides gras. Le rapport $^{3}H/^{14}C$ s'élève ensuite dans les trois tissus en même temps que diminuent toutes les activités brutes. Ceci indique que la pénétration est en grande partie terminée et que le ^{14}C du citrate subit une combustion plus rapide que l'^{3}H de l'acétate, surtout dans le muscle et dans le foie. La majeure partie du citrate qui a tout d'abord pénétré dans le tissu adipeux ne brûle probablement pas dans ce tissu.

(3) La faible incorporation du citrate pourrait également s'expliquer à première vue de la manière suivante: le citrate, après avoir pénétré dans le cytoplasme, échapperait à l'action de l'enzyme clivant et serait ainsi perdu pour la synthèse des acides gras. En effet, les premières étapes du cycle tricarboxylique, qui permettent de transformer le citrate en α-cétoglutarate, peuvent se dérouler dans le cytoplasme (refs. 26, 27). L'interprétation des résultats ne s'en trouverait cependant pas modifiée car si cette voie était compétitive, cela signifierait encore que l'enzyme clivant le citrate est peu actif. Cette hypothèse ne s'accorderait pas d'ailleurs avec l'accroissement, en fonction du temps, de la radioactivité des acides gras observé après admini-

stration de citrate (résultats non publiés). Cette dernière observation signifie donc que puisque le citrate est resté présent dans la cellule, l'activité de l'enzyme clivant est très faible *in vivo*, comme cela a déjà été suggéré par FOSTER ET SRERE[14].

Si nous comparons maintenant l'incorporation du citrate à celle de l'acétate dans les différents acides gras, nous voyons que dans la carcasse de l'animal vivant, le citrate est utilisé de préférence à l'acétate pour la synthèse de l'acide stéarique. Or en présence de surnageant de foie de pigeon, le citrate participe avant tout à la synthèse de l'acide palmitique[3]. Ces différences nous ont incités à penser que ce n'est pas la partie du citrate qui donne naissance à l'acétyl-CoA qui est mieux incorporée dans l'acide stéarique, mais plus vraisemblablement celle qui correspond à l'oxaloacétate. On a en effet de la peine à admettre que les deux acétates, celui administré comme tel et celui provenant du citrate, se comportent différemment. Pour vérifier cette hypothèse, nous avons injecté du [4-^{14}C]aspartate qui, comme le [1,5-$^{14}C_2$]citrate produit de l'oxaloacétate marqué en position 4. L'oxaloacétate pourrait participer à la synthèse des acides gras en empruntant la voie décrite par KALLEN ET LOWENSTEIN[27] (Fig. 4), et selon laquelle l'oxaloacétate peut, par une décarboxylation oxydative, se transformer directement en malonyl-CoA. Nous n'avons trouvé qu'une faible incorporation de l'aspartate dans les acides gras. Ce fait n'a rien d'étonnant, puisque cette voie ne peut être qu'accessoire, ce précurseur ayant d'autres destinées, notamment la synthèse des protéines. D'autre part la voie proposée exige une désacylation puis une réacylation symétrique, réactions vraisemblablement très peu importantes. Il ressort cependant de ces résultats que dans la carcasse, la synthèse de l'acide stéarique est favorisée à partir d'aspartate comme elle l'est à partir du citrate. Cela laisserait sous-entendre que la voie directe oxaloacétate → malonyl-CoA fournit préférentiellement le malonyl-CoA nécessaire à l'élongation microsomale.

RÉSUMÉ

Des souris reçoivent du [1,5-$^{14}C_2$]citrate, du [2-^{3}H]acétate ou encore du [4-^{14}C]-aspartate, soit en injections intraveineuses, soit par intubation. La radioactivité des acides gras du foie, de la carcasse, de l'intestin et du rein est mesurée, ainsi que celle des acides gras des différentes fractions cellulaires du foie. L'activité spécifique totale du foie, du tissu adipeux et du muscle est également mesurée au cours du temps. Le [1,5-$^{14}C_2$]citrate est peu incorporé, comparativement à l'acétate dans les acides gras de la carcasse et du foie. Dans la carcasse, le citrate est utilisé de préférence à l'acétate pour la synthèse de l'acide stéarique; il se produit le même phénomène à partir du [4-^{14}C]aspartate. Le [1,5-$^{14}C_2$]citrate et le [2-^{3}H]acétate participent dans les mêmes proportions à la synthèse des acides gras dans le surnageant et les microsomes; l'incorporation de l'acétate est favorisée dans les mitochondries. Les rapports ^{3}H/^{14}C des activités brutes du foie, du tissu adipeux et du muscle observés après administration de [2-^{3}H]acétate et de [1,5-$^{14}C_2$]citrate croissent tous en fonction du temps. Le rôle du citrate comme précurseur dans la synthèse des acides gras est discuté sur la base de ces résultats.

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Effect of Anticoagulant and ABO Incompatibility on Recovery of Transfused Human Platelets

By RICHARD H. ASTER

PLATELET-RICH PLASMA and platelet concentrates are frequently effective in the treatment of hemorrhage secondary to thrombocytopenia.[1,2] The recovery of transfused platelets in the recipients is often low, however, the average ranging from 20–33 per cent in several series.[3,5,6]

Previous work in this laboratory has shown that a citrate anticoagulant which buffers platelet-rich plasma (PRP) at pH 6.5 improves the viability of concentrated platelets by facilitating their resuspension without clumping.[7] The same study suggested that EDTA, a substance commonly used for platelet transfusions, was toxic for these cells. This work implied that acid citrate was a superior anticoagulant for platelet transfusion therapy, but special manipulations were required to label the platelets with Cr^{51}. In the present study, the effects of different anticoagulant solutions on platelet viability were investigated, utilizing technics comparable to those employed under clinical conditions.

Although A and B blood group antigens have been shown to exist on the platelet membrane,[8-10] their importance for platelet transfusion therapy has not been clarified. It has been suggested that the recovery of group A or B platelets transfused to group O recipients is equal to that of group O donors.[6] Other workers found a "slight, but consistently lower" recovery of "incompatible" platelets.[11] In part, difficulty in assessing the role of ABO antigens in platelet transfusions may have arisen from the relatively low maximum platelet recovery achieved in most studies. The availability of a Cr^{51}-labeling technic that gives a high recovery of platelets[7] permits differences in the recovery of incompatible platelets to be more readily apparent. The use of normal subjects as platelet recipients eliminated variables such as hemorrhage and isoimmunization which might obscure the effect of ABO incompatibilty in ill, thrombocytopenic subjects.

MATERIALS AND METHODS

Platelets were labeled with Cr^{51} and their recovery in recipients was calculated as described previously,[7] with the following exceptions:

1. In most studies the anticoagulant mixture was prepared by adding 0.15 molar citric

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acid directly to the collecting bag containing ACD formula A, rather than preparing fresh anticoagulant on each occasion. The amount of citric acid which produced the required pH of 6.5 when 500 ml. of whole blood was added to the anticoagulant mixture varied with the hematocrit of the donor due to the high buffering effect of red blood cells (RBC). The volume of citric acid required is given as a function of the donor hematocrit in figure 1.* Citric acid was autoclaved and stored at 5 C. for up to 6 months. The average recovery and range of recoveries of autologous platelets in vivo was the same as when freshly prepared anticoagulant was used.

2. During the labeling procedure, platelets were centrifuged at 14 C. rather than at room temperature.

3. After incubation of platelets with Cr^{51}, centrifugation and removal of the supernatant plasma containing excess Cr^{51}, the platelet button was gently covered with 5–10 ml. of platelet-poor plasma (PPP). This plasma in turn was poured off in order to remove remaining Cr^{51} not attached to cells. Platelets were then resuspended in PPP and injected. On the average, 95 per cent of injected Cr^{51} was attached to platelets.

4. High specific activity of platelets used for serial-transfusion experiments (see "Results") was achieved by incubating the concentrated platelets from 500 ml. of blood in a small (10–15 ml.) volume of plasma and adding 1 millicurie of Cr^{51} rather than the usual 300 microcuries. The Cr^{51}, which is suspended in distilled water in the commercial preparation, was made isotonic by the addition of sterile 3 per cent sodium chloride before adding to platelets. In this way 50–100 microcuries of Cr^{51} could be incorporated into transfused platelets.

Recoveries of transfused platelets were based on the average of at least 2 determinations done 1–4 hours after transfusion except with "EDTA platelets,' where the maximum value was used. This maximum sometimes occurred 24 hours after injection.

In some studies of the effect of ABO-incompatibility on platelet recovery, labeled platelets were divided into 4–6 aliquots which were given to an equal number of recipients. In this way the recovery of the same labeled platelet preparation could be compared in both ABO-compatible and ABO-incompatible recipients. When aliquots of the same platelet preparation were given to different ABO-compatible recipients, the range of recoveries was the same as for autologous platelets.

Body surface scanning was performed as previously described.[7,12] Units of surface radioactivity (fig. 4) were standardized by dividing the number of counts per minute observed at the surface by 10 times the number of microcuries of Cr^{51} injected.

Isoagglutinin titers were determined by incubating 1 drop of 2 per cent washed RBC's with 3 drops of serially diluted serum for 30 minutes at room temperature. Agglutination was read after centrifugation for 20 seconds in a serologic centrifuge.

Subjects studied were normal volunteers or volunteer convalescent patients on the medical wards who did not have hematologic disease or diseases of the liver or spleen. Twenty-four of the 41 platelet recipients had not been transfused previously. The remainder had not received more than two transfusions each, and none had received blood during the previous two months. There was no difference in the transfusion history of subjects receiving ABO-incompatible platelets in comparison with those receiving compatible platelets.

Results

Effect of Anticoagulant on Recovery of Transfused Platelets

Autologous platelets labeled with 50–100 μc. of Cr^{51} in the acid citrate anticoagulant were reinfused into the same normal donors. One day later, when 50

*Concentration of citrate or dextrose does not appear to be crucial for satisfactory results, but a pH of 6.5 is necessary before centrifugation of platelet-rich plasma. This can be achieved in a number of ways, including addition of acid to PRP after separation of red blood cells.

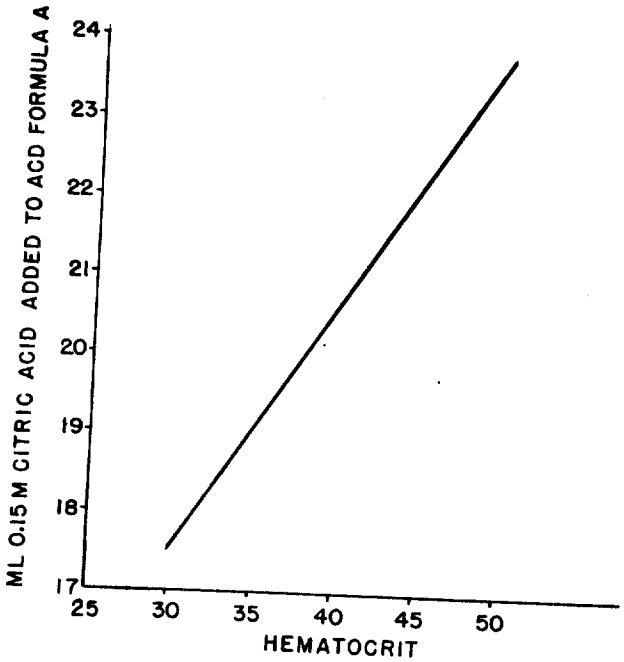

Fig. 1.—Relationship between donor hematocrit and ml. of 0.15 M citric acid added to 75 ml. NIH formula A ACD solution which produced pH about 6.5 in platelet-rich plasma. Values are for 500 ml. of donor blood.

to 63 per cent of the transfused platelets were still circulating (initial recoveries ranged from 55 to 70 per cent), 500 ml. of whole blood was obtained using the following anticoagulants:

a. 50 ml., 1.5 per cent EDTA in 2 per cent dextrose, final pH of platelet-rich plasma (PRP) 7.3 (3 subjects).
b. 95 ml., 0.15 M trisodium citrate in 2 per cent dextrose, final pH of PRP 7.4 (1 subject).
c. 75 ml., NIH formula A ACD plus 22 ml. 0.15 M citric acid, final pH of PRP 6.5 (1 subject).

Using procedures normally employed in platelet transfusion therapy, the whole blood was centrifuged at 1300 r.p.m. for 15 minutes and the PRP was transferred to a separate pack. An aliquot was removed for determination of Cr^{51} activity and the remaining PRP, containing 2–5 μc of Cr^{51}, was transfused to a normal ABO-compatible recipient. Total time between phlebotomy and infusion of PRP was one hour. Platelet recoveries and survival curves are shown in figure 2. With EDTA, most platelets were transiently sequestered after transfusion, and maximum recovery was 34 per cent. With either acid or neutral citrate there was no temporary sequestration, and 70 and 75 per cent of transfused platelets remained in the general circulation in the respective studies. There was no significant difference in platelet recoveries at 1 hour as opposed to 4 hours. These recoveries calculated from the platelet Cr^{51} activity in venous blood probably represent true recoveries of 90–100 per cent

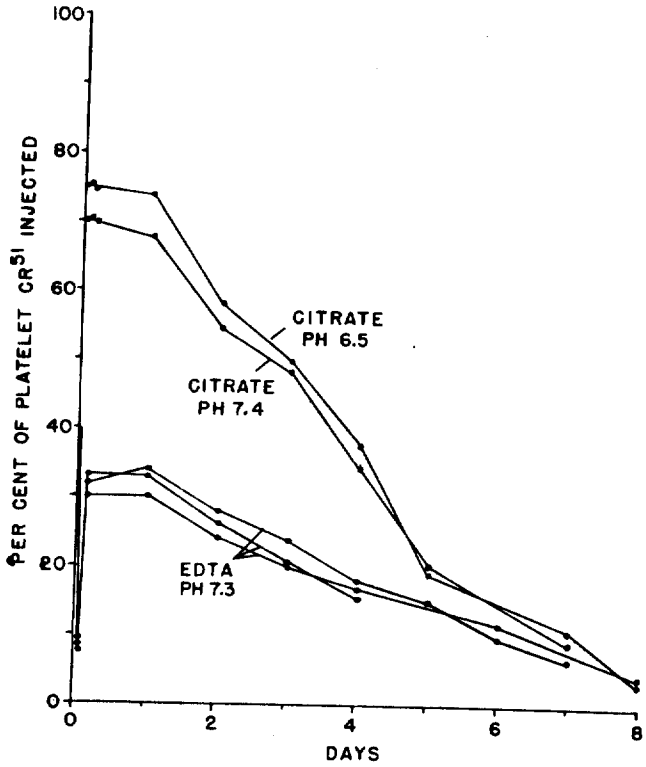

Fig. 2.—Effect of anticoagulant on recovery and survival of Cr^{51}-labeled platelets transfused as platelet-rich plasma without concentration. The pH is that of the platelet-rich plasma after separation from RBC.

of the transfused cells, for 20–30 per cent of platelets appear normally to enter a splenic platelet pool.[13] In all subjects platelet disappearance was approximately rectilinear with time over the next 7 to 8 days.

Effect of ABO Incompatibility on Platelet Recovery

Recoveries of platelets given to ABO-compatible versus incompatible recipients are given in figure 3. In compatible recipients recoveries were about the same as those observed with autologous platelets (average 63 per cent). When platelets from group A_1 donors were given to group O recipients, the average recovery was much lower (19 per cent). When group B platelets were given to incompatible recipients, the average recovery was 57 per cent. When group A B platelets were given to two group O recipients, the average recovery was only 8 per cent. In two subjects, a second transfusion of group A_1 incompatible cells given one week and four weeks later, respectively, resulted in the same low recovery as did the first transfusions. Thus, there was no evidence that "tolerance" following the first antigenic exposure improved the recovery of subsequently injected incompatible platelets.

The removal of a large fraction of ABO-incompatible platelets from the circulation occurred within the first 10 minutes after transfusion, too rapidly

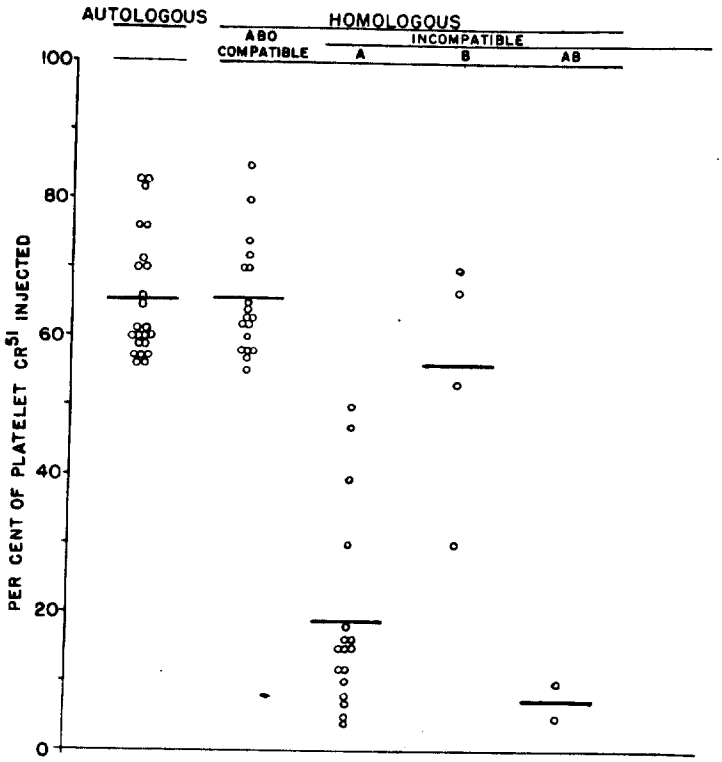

Fig. 3.—Effect of donor ABO group on recovery of transfused Cr^{51}-labeled platelets.

to be accounted for by purely splenic sequestration.[14] There was no corresponding increase in plasma radioactivity to suggest that elution of the Cr^{51} label might have occurred rather than actual destruction of the platelets themselves. Body surface scanning in three patients showed an increase in hepatic surface radioactivity after the infusion of A_1 incompatible platelets which was greater than that observed in any patient after the infusion of ABO-compatible cells (fig. 4). No significant increase was observed over the lungs. With the scanning device used in these studies, a given increase in surface counts per minute (cpm) over the liver represents 3–4 times as much Cr^{51} deposited in the organ as does the same increase in surface cpm over the normal-sized spleen.[7] The increases in hepatic radioactivity after transfusion of ABO-incompatible platelets suggest that most of the platelets were removed from the circulation in the liver. The reduced surface radioactivity over the spleens of these subjects presumably was due to the smaller amount of platelet Cr^{51} remaining in the general circulation and contributing to the splenic platelet reservoir.[13]

After the initial rapid destruction of a fraction of the ABO-incompatible cells, the remaining platelets usually disappeared from the circulation normal-

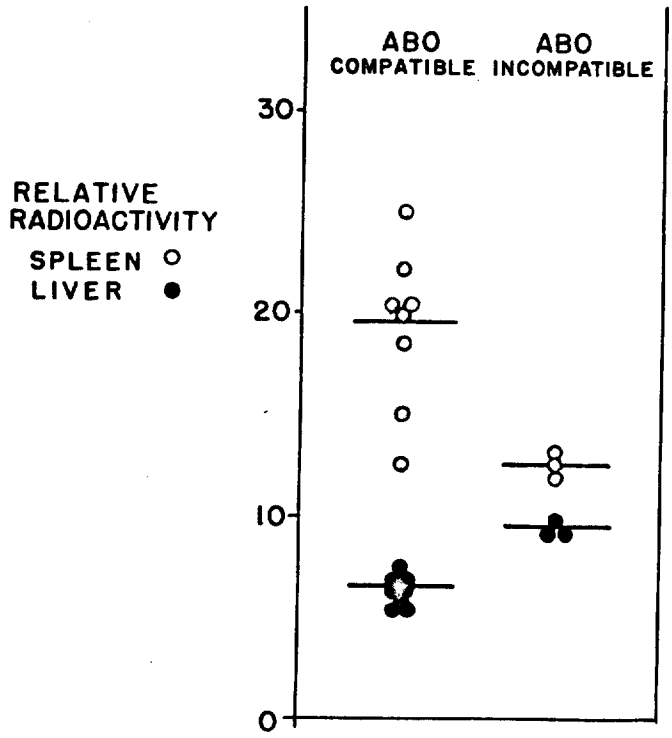

Fig. 4.—Organ surface radioactivity after transfusion of ABO-compatible and incompatible platelets. Units on the ordinate are adjusted so that values are independent of the dose of radioactivity administered.

ly over the next 8 days (fig. 5). In two instances, slightly shortened survival times were presumed due to previous isoimmunization.

The higher recoveries observed after transfusion of group B incompatible platelets suggested that the level of isoagglutinins might be a factor determining platelet recovery, since anti-B isoagglutinins are usually of lower titer than anti-A isoagglutinins. Figure 6 shows that a rough correlation of this sort did, in fact, exist. In general, recoveries were low in recipients with high titers of anti-A or anti-B. Conversely, relatively high recoveries were found in recipients with low isoagglutinin titers. When group A_1B platelets were transfused to group O recipients, the anti-A and anti-B isoagglutinins appeared to act additively in destroying transfused platelets. No relationship could be established between isohemolysin activity and recovery of incompatible platelets.

The normal survival manifested by platelets which survived the period immediately after transfusion suggested that some transient reaction between the infused cells and the recipient's plasma at or near the site of injection might be responsible for the destruction of a portion of the transfused cells. For example, the small number of RBC present in the platelet concentrate would be expected

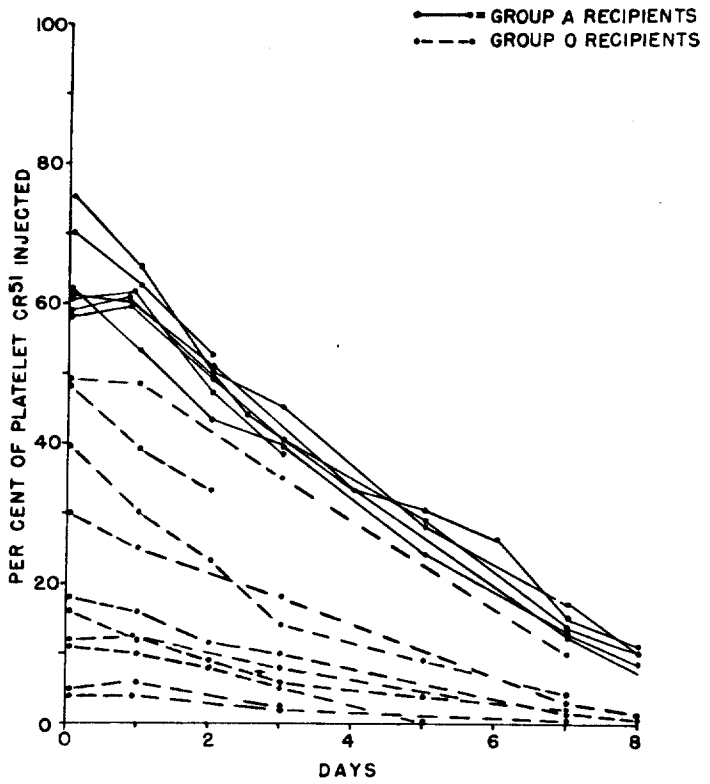

Fig. 5.—Survival of group A_1 platelets in group O recipients. (Dashed lines) Initial recoveries are low, but platelets remaining in the circulation disappeared at the normal rate in most subjects.

to react immediately with isoantibody after entering the recipient's circulation, and might cause platelets to become involved in mixed cell aggregates or might activate plasma factors deleterious to platelets. Several experiments were performed to study these possibilities. In one group O normal subject, autologous platelets were labeled with Cr^{51}. 0.25 ml. of washed red cells from a group A_1 donor was then added to the suspension of labeled platelets before they were reinfused into the original donor. Platelet recovery (58 per cent) and survival was identical to that seen in the same subject without added RBC. In another group O subject, the same experiment was performed except that purified A substance,* derived from hog stomach and equivalent to approximately 4 ml. of packed group A, RBC in antigen content (as determined by in vitro neutralization of anti-A), was substituted for the group A_1 red cells. Again, no decrease in recovery was observed. On two other occasions, A substance equivalent to 10 ml. of packed RBC was infused to group O recipients whose platelets had been labeled one day previously with Cr^{51}, and reinfused. In these sub-

*Obtained from Michael Reese Research Foundation, Chicago, Illinois.

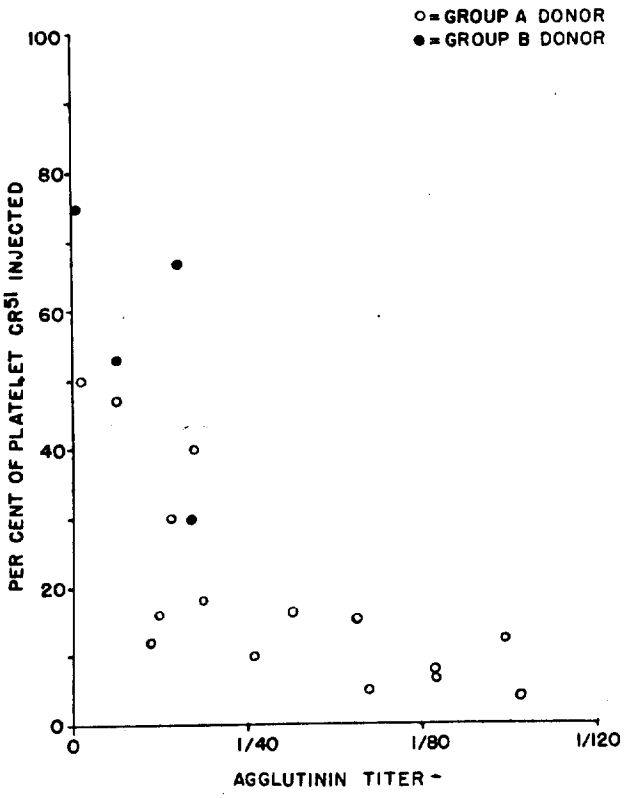

Fig. 6.—Relationship between recovery of ABO-incompatible platelets and homologous isoagglutinin titers.

jects, both circulating Cr^{51} activity and total circulating platelets were reduced by about 25 per cent of the preinjection levels. Simultaneously there was a small but significant rise in hepatic surface radioactivity, but no change over the spleen. The survival time of the remaining labeled platelets was not shortened.

Discussion

Since the studies of Dillard and coworkers in dogs and guinea pigs,[1] EDTA has been used frequently as an anticoagulant for platelet transfusions. It is clear that this agent is satisfactory for the preparation of platelets from most animal species. Early workers suggested that EDTA might be the anticoagulant of choice for platelet transfusions in man, reporting platelet recoveries of 65 per cent[2] and 75 per cent[3] with transfused PRP, and 25 per cent[3] and 52 per cent[2] with concentrates.

The present data indicating that EDTA reduces platelet recovery in man were obtained by using a Cr^{51} label. It has been shown, however, that the Cr^{51} technic does measure the true recovery of transfused human platelets,[7] and the results obtained are in very close agreement with those of Kissmeyer-Nielsen and Madsen,[15] who used an in vivo phosphate label. The latter workers

found that platelet recovery after 3 transfusions of EDTA whole blood was 30 per cent, about one-half the recovery obtained when blood was anticoagulated with ACD. Recently, Freireich and coworkers have confirmed by direct platelet counts after transfusion of concentrates, that EDTA reduces the viability of transfused platelets to about one-half that achieved with citrate at pH 6.5.[16]

Figure 2 shows clearly that EDTA does reduce the viable fraction of transfused platelets to about half that obtained with citrate anticoagulants. Both neutral citrate and acid citrate gave comparable recoveries. Thus, the reduced recoveries with EDTA result from a direct toxic effect of this substance, rather than from the effect of handling platelets at a neutral rather than an acid pH. The number of observations are few, but the differences observed are so striking and so clearly consistent with previous work[7,15] that it was felt that further experiments were not justified in view of the risks inherent in cross-transfusion studies. It is also apparent that EDTA is chiefly responsible for the temporary sequestration of platelets during the first few hours after transfusion, a phenomenon that has been assumed to be an inevitable consequence of handling platelets in vitro, and which is due to trapping of these cells in the liver.[7,17] Thus, during the first few hours after transfusion to a thrombocytopenic recipient, circulating levels of platelets prepared in citrate may be expected to be 5–10 times greater than when platelets are prepared in EDTA. Figure 2 shows that if platelets are not concentrated, the pH of the citrate anticoagulant is of little consequence. If concentration is required, however, it is impossible to resuspend platelets in citrate at pH 7.4 because of adhesion between cells. With the usual ACD solution, which buffers platelet-rich plasma at about pH 7.1, suspension is easier, but aggregation, at least on a microscopic scale, remains a problem and reduces recoveries. At pH 6.5, concentrated platelets can be resuspended readily without detectable clumping.[7] Recoveries of such platelets, even after two centrifugations and resuspensions, ranged from 50–80 per cent (fig. 3).

Preliminary studies strongly suggest that the ease with which concentrated platelets can be resuspended at pH 6.5 is due to interference with the action of adenosine-diphosphate (ADP), which is known to increase platelet adhesiveness at very low concentrations,[18] and which is probably present in shed blood as a result of cell injury. At pH 6.5, the amount of ADP required to cause platelet aggregation in platelet-rich plasma is about 100 times that required at pH 7.4.[19]

The mechanism by which EDTA reduces viability of human platelets remains unclear. It is known that platelets rapidly assume a spherical configuration in EDTA,[20] suggesting that this substance may interfere with ion and water transport across the cell membrane. Ironically, this very effect which smoothes the platelet's shape may have stimulated the use of EDTA as an anticoagulant for transfusion purposes.

The Effect of ABO Incompatibility on Platelet Recovery

It is known that ABO incompatible platelets which remain in the circulation after transfusion *survive* normally.[11,21] The effect of incompatibility on platelet

recovery has not been extensively studied, although Baldini, Costea, and Ebbe noted "slight, but consistently lower recoveries" of ABO-incompatible platelets in an undisclosed number of subjects.[11] Freireich et al. found no effect of ABO groups on platelet recoveries in one patient under treatment for leukemia.[6] Figure 3 indicates that incompatibility with respect to the ABO antigen system is capable of reducing the recovery of transfused platelets. This is particularly true when platelets from group A_1 or group A_1B donors are given to incompatible recipients. When platelet preparations were subdivided and given to several recipients, the dose of platelets was less than that which would be administered in the treatment of thrombocytopenia. However, on six occasions when an entire unit of platelets ($5 \times 10^{10} - 8 \times 10^{10}$ platelets) was transfused to a single recipient, the same low recoveries of incompatible cells were observed as when smaller numbers of platelets were used. Thus, the recoveries in figure 3 do not appear related to platelet dose. Figure 6 indicates that the recovery of incompatible platelets is inversely related to the corresponding isoagglutinin titer. It is apparent that this relationship is not a linear one.

The mechanism by which a fraction of ABO-incompatible platelets are destroyed is not yet clear. The normal survival of cells that escape destruction during the first minutes after transfusion suggests two possible explanations: (1) "A" substance may elute from a portion of the cells after transfusion or may be distributed nonhomogeneously on the platelets themselves. (2) Platelets may be secondarily injured following the reaction between red blood cells or "A substance" present in the transfusion mixture and isoantibody in the recipients' plasma. The first alternative seems unlikely since "A substance" is very stable on platelets in vitro,[10] and, if the label were unevenly distributed among cells, it would be surprising to find a relationship between antibody titer and platelet recovery. The second alternative gains support from the fact that platelets may be injured by the presence of unrelated antigen-antibody complexes in their vicinity.[22-26] The present studies do not reveal the mechanism by which the recovery of ABO-incompatible platelets is curtailed. It is of interest, however, that infusion of "A" substance to group O recipients does produce hepatic sequestration of platelets and that a fraction of ABO-incompatible platelets also were destroyed in the liver after infusion (fig. 4). It may be that failure of "A" substance or group A_1 RBC added to group O platelets to affect recovery of such platelets relates to the fact that a limited range of antigen-antibody ratios produces a secondary type of platelet damage.[23,25]

Implications for Platelet Transfusion Therapy

The present study indicates that for maximum effectiveness, ABO-compatible platelets prepared in a citrate anticoagulant should be used in the treatment of thrombocytopenia. If platelet-rich plasma is transfused, the composition of the citrate medium appears unimportant. If platelets are to be concentrated, the the addition of sufficient citric acid to reduce the pH of platelet-rich plasma to about 6.5 will facilitate platelet resuspension and improve platelet recoveries.

It must be kept in mind that in thrombocytopenic patients, recoveries of transfused platelets may be reduced by other factors such as isoimmunization, hemorrhage, or the patient's disease process itself.[6]

Summary

The effects of anticoagulant solutions on the recovery of transfused platelets were studied. Citrate anticoagulants at pH 7.4 or pH 6.5 were found to be equally effective in preserving the viability of platelets when centrifugation of the cells was not required. When centrifugation is required, as in most platelet survival studies, citrate at pH 6.5 gives maximum recoveries. Ethylenediamine tetraacetate (EDTA) caused temporary sequestration of nearly all transfused platelets and reduced maximum recoveries by about 50 per cent.

Platelet recovery was lowered by ABO-incompatibility between donor platelets and recipient serum, but survival time of remaining platelets was not altered. Lowest platelet recoveries resulted when group A_1 or A_1B platelets were given to group O recipients with high isoagglutinin titers.

It is suggested that ABO-compatible platelets prepared in citrate should be used where possible in the treatment of thromboctyopenic disorders.

Summario in Interlingua

Esseva studiate le effectos de solutiones anticoagulante super le restablimento de transfusionate plachettas. Esseva constatate que anticoagulantes citratic a pH 7.4 o pH 6.5 esseva equalmente efficace in preservar le viabilitate de plachettas quando centrifugation del cellulas non esseva requirite. Quando centrifugation es requirite—como es le caso in le majoritate del studios de superviventia plachettal—citrato a pH 6.5 resulta in un restablimento maximal. Tetra-acetato ethyleno-diaminic (EDTA) causava un sequestration temporari de quasi omne le transfusionate plachettas e reduceva le maximo de restablimento per circa 50 pro cento.

Le restablimento plachettal esseva reducite per incompatibilitate typo ABO inter le plachettas del donator e le sero del recipiente, sed le tempore del superviventia del remanente plachettas non esseva alterate. Le plus basse mesura de restablimento plachettal esseva observate quando plachettas de gruppo A_1 o A_1 B esseva donate a recipientes de gruppo O con alte titros isoagglutininic.

Es recommendate que plachettas a compatibilitate in ABO preparate in citrato debe esser usate in tanto que possibile in le tractamento de disordines thrombocytopenic.

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Metabolic response of prematures to milk formulas with different lactic acid isomers or citric acid

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When, some years ago, Goldman et al. [1] described metabolic acidosis in prematures receiving a milk acidified by lactic acid, it was hypothetically supposed that the acidosis could be due to the fact that the D(−) isomer is not metabolised.

Various clinical and biochemical studies carried out on commercial milks acidified by lactic acid (Ofteringer [2], Hungerland [3], Vesterdal [4], Ballabriga [5–6]) showed that such milks did not cause clinically discernible acidosis and that the acid-base balance values remained within normal limits. Cevini [7] encountered metabolic acidosis using racemic lactic acid.

The aim of this study is to examine the metabolic and clinical responses of prematures fed various milk formulas with varying protein contents and acidified with racemic acid, L(+) isomer, D(−) isomer or a large amount of citric acid.

Material and methods

The study was made on 150 premature infants, all of whom were considered to be "normal" from the clinical point of view, without any signs of distress. The biochemical investigations were carried out from the 12th day of life when calory intake had reached a level of 120 cal./kg bodyweight/day, it being considered that beforehand even the normal premature infant may show considerable alterations in acid-base balance, such alterations being caused by his adaptation to his new surroundings.

A micro-Astrup apparatus, model Amei, was used to study the acid-base balance and the values were calculated with the new pH-log pCO_2 nomogram revised by Sigaard-Andersen [8] in 1962.

All the blood samples were taken from the heel, using heparinized capillary tubes. A double determination was made in every case and the blood samples were taken at the same time of day in order to avoid any possible variations. The double determinations were considered to be valid when the actual pH values or the pH values after saturation of the samples, did not differ by more than 0.005.

In those cases in which the blood sample was taken whilst the infant was crying loudly, the double determination was not accepted. The analysis was carried out at once, after the sample had been shaken. A total of 600 double determinations were made, the actual pH, pCO_2, standard bicarbonate and base excess values being calculated.

Table 1

Composition of the formulas used, in g/100 ml

Formula	N	X	X+D−	X+L+	X+ racemic	E	X+ citric	N+ citric
Fats	3.4	1.4	1.4	1.4	1.4	1.3	1.4	3.4
Proteins	1.62	3.19	3.19	3.19	3.19	2.9	3.19	1.62
Lactose	7.34	4.43	4.43	4.43	4.43	3.8	4.43	7.34
Starch	0	0	0	0	0	1.6	0	0
Dex. maltose	0	6.0	6.0	6.0	6.0	4.8	6.0	0
Mineral salts	0.2	0.64	0.64	0.64	0.64	0.6	0.64	0.2
Lactic acid	0	0	0.5D−	0.5L+	0.5R	0.5L+	0	0
Citric acid	0	0	0	0	0	0	0.5	0.5
Calories	68.25	68.8	68.8	68.8	68.8	66.0	68.8	68.25
pH	6.65	6.71	4.98	5.1	4.9	4.6	4.8	4.1

The 150 premature infants who served as the object of this study were fed 8 different types of foods and the differences in the composition of these products are given in Table 1.

All the subjects regularly received an equivalent of 120 cal./kg bodyweight/day from the age of 12 days, irrespective of the product administered. The food was administered by stomach tube or as a bottle feed, a total of 8 feeds per day were given. Vitamin C daily intake was 60 mg/kg.

All the prematures received the non-acidified formula N until aged 12 days, then the following diets: formula X: 25 cases; formula X with L(+) lactic acid: 21 cases; formula X with D(−) lactic acid: 16 cases; formula X with racemic lactic acid: 29 cases; formula E with L(+) lactic acid obtained by biological acidification: 21 cases; formula X with citric acid: 16 cases; formula N with citric acid: 22 cases.

Each infant received the diet for 12 days during which time four determinations of the acid-base balance were taken; the first whilst the infant was still receiving formula N without lactic acid: on the same day he was transferred to one of the experimental acidified formulas and the acid-base balance was taken on the 4th, 8th and 12 days of this diet.

The D(−) lactic acid used was the one produced by Kyowa Hakko Kogyo (Japan) at 52%, of which 70% was the D(−) form. A 30% D(−) lactic acid free acid from the Sigma Chemical Co., St. Louis, was also used.

80% racemic lactic acid was obtained from Koge Chemical Works, Copenhagen and L(+) lactic acid from Boehringer Sohn, Ingelheim. The Chemische Fabrik Schweizerhall, Basel, supplied the citric acid (lead free).

The amounts of calories, proteins, liquid and acid administered per kg bodyweight/day with these diets were as follows: see Tab. 2.

Urinary elimination of organic acids was studied only in the boys of each group by twodimensional chromatography. The urine samples for 24 h were collected and put in a refrigerator until analysis. Finally, 75 prematures were chosen and four determinations were made on each of them, the first at the beginning of the experiment whilst they were still receiving the acid free N Formula (sample no 1) then on the 8th and 12th day of the diet containing the different lactic and citric acids (samples nos 2 and 3). The acidified milk diet was stopped after 12 days and the Formula N diet regiven for a period of 10 days after which the final determination of eliminated organic acids was made (sample no 4).

Table 2

	Calories	Calories % from proteins	Liquid cm³	Proteins g	TA* mEq/kg
Formula N	120	9.7	175	2.84	1.03
Formula X	120	18.9	174	5.5	1.35
Formula X with D(−)	120	18.9	174	5.5	9.37
Formula X with L(+)	120	18.9	174	5.5	8.09
Formula X with racemic	120	18.9	174	5.5	9.15
Formula E	120	17.0	175	5.0	9.03
Formula X with citric	120	18.9	174	5.5	10.61
Formula N with citric	120	9.7	175	2.84	10.62

*TA = Titrable acid expressed in mEq/kg/day obtained by titrating the milk to end point pH 7.4 using the radiometer automatic titrator TTT 1.

The NORDMAN and NORDMAN [9, 10] method of twodimensional chromatography was used with a solvent of alcohol/ammonia/water 400:25:75 and eucalyptol/propanol/formic acid/water 200:200:80:18. Organic acids were identified by comparison with internal standards and studying the run, fluorescence and colour in front of various dye solutions. Acridine and bromocresol green were generally used as dyes and either p-aminobenzoic aldehyde, iodised starch, silver nitrate, p-nitroaniline or sulphanilic acid as develop reagents.

When the chromatogram showed abnormal pattern, ketonic acids in the same urine samples was measured using the CAVALLINI and FRONTALI [11–13] method to detect the possible presence of p-hydroxyphenyl-pyruvic acid. One-dimensional chromatography with the following two solvents was used:

no 1: amylic alcohol/absolute alcohol/water saturated (40:10)

no 2: butanol/ethanol/water 100:25:75, to detect ketogluxaric acid run in the same direction.

We studied the phenolic acid elimination by the ARMSTRONG [14] method adapted to one-dimensional chromatography using benzene/propionic acid/water 200:140:10 as solvent. Results were interpreted by internal standards and specific developers to confirm the existence of p-hydroxyphenyl-lactic and p-hydroxyphenyl-acetic acids.

When a rise in phenolic acid excretion was observed the urinary tyrosine elimination was analysed by high tension electrophoresis: 7,000 volts for 45 min, pH 1,9 (Holzel Technik Apparatus, Munich) [15], ninhydrine and Pauly reagents for tyrosine were used as staining methods. Statistical treatment of data have been made with a Sharp I.C. Compet 32 electronic desk calculator.

Results

The study of the acid-base balance in each group gave the following results:

a) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 25 prematures fed formula N, then the non acidified formula X for 12 days.

$\bar{x}$ pH Formula N = 7.352 $s = \pm 0.04$ $s_{\bar{x}} = \pm 0.008$

$\bar{x}$ pH Formula X = 7.339 $s = \pm 0.037$ $s_{\bar{x}} = \pm 0.007$

p between 0.3 and 0.2

$\bar{x}$ St. B. Formula N = 22.12 $s = \pm 2.29$ $s_{\bar{x}} = \pm 0.45$
$\bar{x}$ St. B. Forpula X = 21.25 $s = \pm 1.94$ $s_{\bar{x}} = \pm 0.38$
p between 0.2 and 0.1

$\bar{x}$ pCO_2 Formula N = 42.4 $s = \pm 3.52$ $s_{\bar{x}} = \pm 0.7$
$\bar{x}$ pCO_2 Formula X = 41.9 $s = \pm 3.84$ $s_{\bar{x}} = \pm 0.76$
p between 0.3 and 0.2

$\bar{x}$ B. E. Formula N = -2.25 $s = \pm 2.9$ $s_{\bar{x}} = \pm 0.53$
$\bar{x}$ B. E. Formula X = -3.54 $s = \pm 3.69$ $s_{\bar{x}} = \pm 0.53$
p between 0.2 and 0.1

b) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 21 prematures who received non acidified formula N followed by a period of 12 days with formula X with L(+) lactic acid.

$\bar{x}$ pH Formula N 7.351 $s = \pm 0.04$ $s_{\bar{x}} = \pm 0.008$
$\bar{x}$ pH Formula X 7.343 $s = \pm 0.028$ $s_{\bar{x}} = \pm 0.006$
with L(+) p between 0.6 and 0.5

$\bar{x}$ St. B. Formula N = 22.9 $s = \pm 2.5$ $s_{\bar{x}} = \pm 0.54$
$\bar{x}$ St. B. Formula X = 22.1 $s = \pm 1.37$ $s_{\bar{x}} = \pm 0.3$
with L(+) p between 0.3 and 0.2

$\bar{x}$ pCO_2 Formula N = 43.76 $s = \pm 4.9$ $s_{\bar{x}} = \pm 1.07$
$\bar{x}$ pCO_2 Formula X = 43.43 $s = \pm 4.1$ $s_{\bar{x}} = \pm 0.91$
with L(+)
p between 0.9 and 0.8

$\bar{x}$ B. E. Formula N = -1.23 $s = \pm 2.82$ $s_{\bar{x}} = \pm 0.61$
$\bar{x}$ B. E. Formula X = -2.36 $s = \pm 1.7$ $s_{\bar{x}} = \pm 0.37$
with L(+) p between 0.2 and 0.1

c) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 16 prematures who received non acidified formula N followed by a period of 12 days with formula X with D(—) lactic acid.

$\bar{x}$ pH Formula N = 7.365 $s = \pm 0.039$ $s_{\bar{x}} = \pm 0.09$
$\bar{x}$ pH Formula X = 7.322 $s = \pm 0.07$ $s_{\bar{x}} = \pm 0.017$
with D(—) $p < 0.05$

$\bar{x}$ St. B. Formula N = 22.68 $s = \pm 2.12$ $s_{\bar{x}} = \pm 0.53$
$\bar{x}$ St. B. Formula X = 20.37 $s = \pm 3.28$ $s_{\bar{x}} = \pm 0.8$
with D(—) $p < 0.05$

$\bar{x}$ pCO_2 Formula N = 42.34 $s = \pm 4.37$ $s_{\bar{x}} = \pm 1.09$
$\bar{x}$ pCO_2 Formula X = 40.12 $s = \pm 5.05$ $s_{\bar{x}} = \pm 1.2$
with D(—) p between 0.3 and 0.2

$\bar{x}$ B. E. Formula N = -1.16 $s = \pm 2.8$ $s_{\bar{x}} = \pm 0.7$
$\bar{x}$ B. E. Formula X = -5.13 $s = \pm 5.08$ $s_{\bar{x}} = \pm 1.2$
with D(—) $p < 0.05$

d) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 29 prematures who received non acidified formula N followed by a period of 12 days with formula X with racemic lactic acid.

$\bar{x}$ pH Formula N 7.36 $s = \pm 0.04$ $s_{\bar{x}} = \pm 0.007$
$\bar{x}$ pH Formula X = 7.331 $s = \pm 0.04$ $s_{\bar{x}} = \pm 0.008$
with racemic $p < 0.02$

$\bar{x}$ St. B. Formula N = 22.95 $s = \pm 2.14$ $s_{\bar{x}} = \pm 0.39$
$\bar{x}$ St. B. Formula X = 21.8 $s = \pm 2.21$ $s_{\bar{x}} = \pm 0.41$
with racemic $p < 0.05$

$\bar{x}$ pCO_2 Formula N = 43.0 $s = \pm 4.2$ $s_{\bar{x}} = \pm 0.77$
$\bar{x}$ pCO_2 Formula X = 44.4 $s = \pm 4.56$ $s_{\bar{x}} = \pm 0.84$
with racemic p between 0.3 and 0.2

$\bar{x}$ B. E. Formula N = -0.72 $s = \pm 2.16$ $s_{\bar{x}} = \pm 0.4$
$\bar{x}$ B. E. Formula X = -2.99 $s = \pm 2.89$ $s_{\bar{x}} = \pm 0.53$
with racemic $p < 0.01$

e) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 21 cases who received non acidified formula N followed by a period of 12 days with formula E containing L(+) lactic acid obtained by biological acidification.

$\bar{x}$ pH Formula N = 7.331 $s = \pm 0.04$ $s_{\bar{x}} = \pm 0.009$
$\bar{x}$ pH Formula E = 7.329 $s = \pm 0.031$ $s_{\bar{x}} = \pm 0.006$
with L(+) p between 0.9 and 0.8

$\bar{x}$ St. B. Formula N = 20.48 $s = \pm 1.75$ $s_{\bar{x}} = \pm 0.38$
$\bar{x}$ St. B. Formula E = 21.05 $s = \pm 1.74$ $s_{\bar{x}} = \pm 0.38$
with L(+) p between 0.3 and 0.2

$\bar{x}$ pCO_2 Formula N = 40.33 $s = \pm 1.42$ $s_{\bar{x}} = \pm 0.3$
$\bar{x}$ pCO_2 Formula E = 41.9 $s = \pm 1.44$ $s_{\bar{x}} = \pm 0.3$
with L(+) p between 0.2 and 0.1

$\bar{x}$ B. E. Formula N = -4.59 $s = \pm 2.43$ $s_{\bar{x}} = \pm 0.53$
$\bar{x}$ B. E. Formula E = -3.85 $s = \pm 2.3$ $s_{\bar{x}} = \pm 0.5$
with L(+) p between 0.4 and 0.3

f) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 16 prematures who received non acidified formula N followed by a period of 12 days with formula X with citric acid.

$\bar{x}$ pH Formula N = 7.331 $s = \pm 0.038$ $s_{\bar{x}} = \pm 0.009$
$\bar{x}$ pH Formula X = 7.281 $s = \pm 0.079$ $s_{\bar{x}} = \pm 0.19$
with C.Ac. $p < 0.05$

$\bar{x}$ St. B. Formula N = 21.81 $s = \pm 2.0$ $s_{\bar{x}} = \pm 0.5$
$\bar{x}$ St. B. Formula X = 18.86 $s = \pm 3.8$ $s_{\bar{x}} = \pm 0.96$
with C.Ac. p between 0.02 and 0.01

$\bar{x}$ pCO_2 Formula N = 44.93 $s = \pm 5.01$ $s_{\bar{x}} = \pm 1.25$
$\bar{x}$ pCO_2 Formula X = 40.94 $s = \pm 5.22$ $s_{\bar{x}} = \pm 1.3$
with C.Ac. $p < 0.05$

$\bar{x}$ B. E. Formula N = -2.81 $s = \pm 2.73$ $s_{\bar{x}} = \pm 0.68$
$\bar{x}$ B. E. Formula X = -6.94 $s = \pm 5.12$ $s_{\bar{x}} = \pm 1.2$
with C.Ac. $p < 0.01$

g) Comparison of the pH, standard bicarbonate, pCO_2 and base excess values in 22 prematures who received non acidified formula N followed by a period of 12 days with formula N with citric acid.

$\bar{x}$ pH Formula N = 7.345 $s = \pm 0.03$ $s_{\bar{x}} = \pm 0.006$
$\bar{x}$ pH Formula N = 7.344 $s = \pm 0.037$ $s_{\bar{x}} = \pm 0.008$
with C.Ac. p between 0.9 and 0.8

$\bar{x}$ St. B. Formula N	= 22.21	$s = \pm 2.02$	$s_{\bar{x}} = \pm 0.44$
$\bar{x}$ St. B. Formula N with C.Ac.	21.77	$s = \pm 2.16$	$s_{\bar{x}} = \pm 0.47$
		p between 0.6 and 0.5	
$\bar{x}$ pCO_2 Formula N	43.47	$s = \pm 3.87$	$s_{\bar{x}} = \pm 0.84$
$\bar{x}$ pCO_2 Formula N with C.Ac.	= 41.87	$s = \pm 2.57$	$s_{\bar{x}} = \pm 0.56$
		p between 0.2 and 0.1	
$\bar{x}$ B. E. Formula N	= −2.11	$s = \pm 2.66$	$s_{\bar{x}} = \pm 0.58$
$\bar{x}$ B. E. Formula N with C.Ac.	= −2.83	$s = \pm 2.68$	$s_{\bar{x}} = \pm 0.58$
		p between 0.4 and 0.3	

We compared the urine chromatograms of the organic, ketonic and phenolic acids to detect the presence of intense tyrosyluria (increase of tyrosine, p-hydroxyphenylpyruvic, p-hydroxyphenyllactic, p-hydroxyphenylacetic acids in the urine) or an increase in the excretion of organic acids, especially lactic acid. The results of this comparison are set out in Table 3. Only the substantial and intense alterations of the chromatograms were taken into consideration.

Development was normal from the clinical point of view in the groups receiving formula N and non acidified formula X, the formulas acidified by L(+) lactic acid (X with L(−) and E), or the formulas with large amount of citric acid.

Amongst the group of premature receiving the D(−) lactic acid formula X, there were four cases with severe clinical signs of metabolic acidosis i.e. loss of weight, pallor, bad overall condition, vomiting and regurgitation which necessitated the discontinuation of the diet and the correction of the acidosis with intravenous bicarbonate infusion.

In the group receiving the formula acidified by racemic lactic acid there were three cases with similar clinical signs but milder which disappeared when the formula was discontinued. The infants receiving formula X with a high citric acid content showed no signs of clinical anomaly inspite of much lower acid-base balance values than those obtained during the non acidified formula N diet.

Table 3

Formula	Protein intake g kg/d	Acid mEq kg/d	Cases	Number of chromatograms	Very intensive tyrosyluria	Organic aciduria	Lactic acid excretion increased
N	2.84	1.03	73	146	0	0	0
E	5.0	9.03	5	10	0	0	0
X	5.5	1.35	10	20	1	0	0
X + lactic acid L(+)	5.5	8.09	5	10	0	0	0
X + racemic	5.5	9.15	10	20	4	2	1
X + cit. ac.	5.5	10.61	11	22	4	0	0
X + lactic acid D(−)	5.5	9.37	7	14	2	1	2
N + citric	2.84	10.62	11	22	0	0	0
N + racemic	2.84	8.83	5	10	0	1	3
N + lactic acid D( )	2.84	9.05	9	18	2	2	2

Discussion

There was no significant difference between the acid-base values of the prematures fed a non acidified relative protein diet (5.5 g/kg/d) and those fed a low protein diet (2.84 g/kg/d).

When the high protein diet was supplemented with L(+) lactic acid or biologically acidified, no significant differences occurred in the pH, pCO_2, standard bicarbonate or base excess values compared with the low protein non acidified formula.

"Biochemical" metabolic acidosis with acid-base balance values significantly lower than those observed with the low or high protein non acidified diets was noted in those groups fed the acidified, high protein, racemic acid or D(−) isomer diet. In some cases the metabolic acidosis was revealed by clinical signs, such as extreme pallor, depression, bad overall condition, loss of weight, regurgitations and vomiting necessitating the discontinuation of the diet.

Biochemical and clinical metabolic acidosis disappeared when a non acidified, low protein diet was administrated, or after intravenous injection of bicarbonate in the very severe cases.

The high protein diet with a large amount of citric acid (10.62 mEq/kg/d) induced biochemical metabolic acidosis without clinical signs, whereas the same amount of citric acid with a low protein diet did not. The manufactured formulas generally contain much less citric acid (approximately 4.3 mEq/kg/d) and do not induce metabolic acidosis.

On the whole a large number of prematures fed high protein diets seem to border on biochemical metabolic acidosis without clinical signs and the administration of a semi-metabolised acid such as racemic lactic acid, or the D(−) isomer, is sufficient to produce complete biochemical metabolic acidosis with or without clinical signs. The same result can occur with doses of citric acid higher than those generally used in manufactured milks. The significant differences between groups receiving the high protein diets with racemic lactic acid or D(−) are attributable to the differences in the degree of metabolisation of the administered isomer.

Brin's [16] experiments on animals using various C14 labelled isomers showed that a significant amount of D(−) lactate was oxidised with a broad maximum peak at about the end of the first hour after injection. Schimassek's [17] experimental studies showed that in the isolated rat liver the rate of D(−) lactate oxidation is nearly the same as the conversion of L(+) lactate. Pyruvate, formed by the oxidation of D(−) lactate from mitochondria D(−) oxidase, is neither immediatley oxidised nor converted to L(+) lactate. Experiments on animals by Drury and Wick [18], who also used C14 labelled isomers, showed that eviscerated tissues metabolise L(+) quite actively and are able to oxidase a limited quantity of the D(−) isomer.

The authors established that racemic lactate is almost completely metabolised by the intact animal and it is probable that the liver converts D(−) isomer either into the L(+) form, glucose or glycogen.

In infants, during the first weeks of life, it is extremely probable that acidosis occurs on administration of racemic or D(−) lactic acid because the isomer is given in excessive amounts and cannot be completely metabolised, due to the fact that the immature liver cannot convert the D(−) isomer into either the L(+) form, glucose or glycogen. This explains why some prematures are perfectly able to tolerate formulas with a fairly D(−) isomer content, whereas others have severe metabolic acidosis. On the other hand, if the infants are fed a high protein diet which may produce a condition approaching biochemical acidosis, the administration of a non-metabolised isomer facilitates the appearance of clinically recognisable acidosis in the majority of the cases. For this reason metabolic acidosis is much less frequently observed in prematures fed a low protein diet, even one acidified by racemic or D(−) lactic acid.

The clinical symptoms of cases with severe metabolic acidosis after administration of D(−) isomer are the same as DUNLOP and HARRIS [19] described in ruminants, i.e. abdominal disorders, dehydration, depression, fall of pH, bicarbonate and progressive increase in the concentration of blood D(−) lactate.

The study of the urinary organic acid elimination revealed three kinds of alterations: a) intensive tyrosyluria: b) increase in organic acid excretion; c) strong elimination of lactic acid.

During the neonatal period the occurrence of tyrosyluria with varying intensity can be attributed to the following factors: a) protein intake; b) vitamin C intake: c) p-hydroxyphenylpyruvate-oxidase activity in the liver.

Amongst the prematures receiving the low protein formula N, the high protein non-acidified formula X or the formula acidified by L(+) lactic acid, there was only one case of intense tyrosyluria (receiving the non-acidified formula X) as opposed to a much higher incidence in the groups receiving the high protein formula with racemic acid D(−) isomer or a large amount of citric acid (Tab. 3). There are, therefore, border line cases of tyrosyluria during the neonatal period in prematures receiving a high protein formula, which frequently develop into intense tyrosyluria when additional incompletely metabolised acids are administered. The latter may have a toxic effect and temporarily inhibit p-hydroxyphenylpyruvate-oxidase activity in the liver. Strong tyrosyluria also occurs, but much less frequently, with low protein diets (2.84 g/kg/d), containing racemic lactic acid or D(−) isomer. During the period of intense tyrosyluria there is a drop in the excretion of acids of the Krebs cycle, but a notable increase occurs when a low protein non acidified diet is administered.

In the groups receiving racemic lactic acid or the D(−) isomer there was a general increase in the urinary excretion of organic acids in some cases, especially in the spot corresponding to lactic acid elimination, representing an excess of non metabolised D(−) isomer. This was not observed in the prematures fed the citric acid formula. The alterations in organic acid elimination observed in the infants receiving the D(−) isomer, with intense metabolic acidosis and dehydration, are difficult to interpret given that there was possible impairment of kidney function. As in the tyrosyluria cases AVERY et al. [20] studied during the neonatal period, we found no correlation between the existence of metabolic acidosis and the intensity or frequency of tyrosyluria, nor between the latter and the presence of depression and lethargy. No clinical signs indicating metabolic acidosis accompany acute tyrosyluria. The cases of intense lactaciduria, fed the D(−) isomer diet had varying degrees of metabolic acidosis.

Summary

Prematures fed milks acidified with racemic lactic acid or D(−) lactic acid during the first two months of life develop "biochemical" metabolic acidosis, frequently accompanied by the following clinical signs: bad overall condition, loss of weight, dehydration, regurgitation and vomiting. The pH, standard bicarbonate and base excess values are significantly lower during the period of the racemic and D(−) isomer diets than with milk acidified by L(+) lactic acid or a non acidified milk with the same protein content.

A diet containing a large amount of citric acid (10.62 mEq/kg/d) could induce biochemical metabolic acidosis without clinical signs.

Tyrosyluria occurred at times in the infants receiving the high protein diets and was much more frequent and intense with the diet containing racemic lactic acid or the D(−) isomer. This intense tyrosyluria does not depend on the presence of metabolic acidosis and is without clinical signs. In some of these cases we observed an increase in the urinary elimination of organic acids, especially lactic acid. The mechanism responsible for these alterations is discussed.

The conclusion to be drawn from this work is that when acidified milks are used in premature feeding, the formulas must contain exclusively L(+) lactic acid or possibly citric acid provided the dose is below 4.5 mEq/kg/d. Racemic lactic acid and D(−) lactic acid should not be used in infant feeding.

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Comparative Study of Metabolic Disturbances in the Liver of the Rat Caused by the Oral Administration and Intraperitoneal Injection of Citrate

by P. Baron, G. Griffaton and R. Lowry(*) (**)

Numerous works in the last ten years have emphasized the important role of citrate as a regulator of energizing metabolism (2) under various physiological conditions (1, 23).

Citrate intervenes as well in the systems for the synthesis of fatty acids (18, 17, 11) in glycolysis (20, 19, 6) and gluconeogenesis (28).

It thus seemed interesting to us to determine the comparative effects of two methods of administering citrate: intraperitoneal injection of 1 g/kg of sodium citrate at pH 7.2, and oral ingestion of 5 g/kg of a mixture of citric acid and a salt of monosodium citrate and monopotassium citrate (***).

For this purpose, we studied the effect of citrate on the large biochemical cellular functions: lipogenesis, ketogenesis, gluconegenesis, oxidation and glycolysis.

We estimated ketogenesis by the sum of the concentrations of β-hydroxybutyrate and acetoacetate. The concentration of α-glycerophosphate is a regulating factor of lipogenesis. We estimated the entire state of oxidation-reduction and the potential for cellular synthesis by the relationship of NADH to total NAD, while the lactate/pyruvate reflects the cytoplasmic relationship and the β-hydroxybutyrate/acetoacetate ratio is an index of the intramitochondrial NADH/NAD ratio, and therefore of the activity of the Krebs cycle. The determination of α-ketoglutarate, glutamate, malate, and oxaloacetate gives additional indications of the functioning of the Krebs cycle, that of oxaloacetate enabling as well, the estimation of gluconeogenesis.

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(***) Composition of the mixture: citric acid, 1.30 g; mono-K citrate, 1.76 g; mono-Na citrate, 1.845 g; trimethoxybenzene, 0.20 g; "yellow sun", 1 mg; Na cyclamate, 115 mg; Na saccharinate, 11 mg; orange juice, 9.2 mg; mandarin juice, 2.5 mg. We are grateful to the Lafon Laboratories for kindly supplying us with this mixture.

Therefore, for this work, we determined the concentrations of cofactors in the liver in the oxidized form (NAD, NADP) and the reduced form (NADH, NADPH) and of the following substrates: lactate, pyruvate, β-hydroxybutarate, acetoacetate, malate, oxaloacetate, citrate, α-Ketoglutarate, glutamate, α-glycerophosphate and dihydroxyacetone-phosphate. This series of determinations was completed by the measurement of the concentration of urea in the liver, and or uremia and glycemia.

Experimental Protocol

Following preliminary experiments in which the citrate mixture was given in amounts of 2.5 g/kg *per os* and for which it was difficult to make any conclusions, we undertook our experiment using stronger doses. Beyond 5 g/kg, it seemed to us that the solution would become thicker, making gastric intubation more difficult. Therefore, we tested the effects of citrate administered *per os* in the amount of 5 g/kg. The experiment was performed on male Wistar rats, 125-135 fasted for 18 hours.

For the measurement of citrate in the gastric cavity and in the blood, we determined in relation to the quantity of the citrate mixture administered, the quantity of citrate recovered and, from the difference, the quantity of metabolized citrate (Table I).

Table I. Percentage of citrate recovered and metabolized after intraperitoneal injection and gavage of citrate. Hepatic levels of citrate under the same conditions. (In parentheses, the number of rats)

	% of mixture of citrate salt & citric acid recovered	% of mixture of citrate salt & citric acid metabolized	Hepatic levels of citrate in nanomoles/g M±Sm	
Control	-	-	88±10,0	(4)
Citrate i.p. 1 g/kg rat killed 30 min later	5	95	1542±50,9	(4)
Citrate *per os* 5 g/kg rat killed one hour later	63	37	257±18,4	(4)
Citrate *per os* 5 g/kg rat killed 2 hrs later	53	47	218±41,2	(3)
Citrate *per os* 5 g/kg rat killed 3 hrs later	36	64	181±20,5	(3)

We made these measurements at the same time that we measured the hepatic concentration of citrate on 4 groups of rats:

- control rats receiving no treatment. A previous study showed that factors other than the citrate ion administered at the time of the i.p. injection or gavage were without effect;
- rats subjected to an i.p. injection of 1 g/kg of citrate and sacrificed 30 minutes after the injection. This lot enabled us to compare the effects of citrate administered by gavage to the rapid and massive effects of citrate administered i.p.;
- rats subjected to a citrate mixture (5 g/kg) by gastric intubation and sacrificed an hour after the intubation, the second, one hour later and the third, 2 hours later.

The table shows that it is not good to wait 3 hours because, although the metabolized quantity increases with time, it is necessary to have concentrations sufficiently different from those of the controls in order to estimate the effects. This is why in the course of the work, we made the determinations of the substrates and the cofactors (see introduction) on the first 4 lots of rats.

For these measurements, we used our own personal modifications of the enzymatic methods published by Bergmeyer (5).

The liver, quickly removed and washed in isotonic KCl was cut into two pieces, one of which was immediately put into liquid nitrogen. The substrates were measured after perchloric extraction with Ultra-Turrax and the elimination of the excess acid. On the other piece of liver, we determined the reduced and oxidized forms of dinucleotides, after extraction of the tissue in a phosphate buffer at boiling.

This did not take into consideration contamination from the blood, nor the actual intracellular space.

We expressed the results in nanomoles or in micromoles per gram of fresh liver. The values obtained were subjected to analysis of the variations. When the variations of the different groups compared form a normally distributed population (verified by the appropriate tests), the analysis of the variation leads to the calculation of a standard deviation common to all these groups. The degree of the significance of the differences between the groups was evaluated by means of the F test.

In the case of calculating the standard deviations from the averages of the ratios, it is necessary to normalize the variation by an angular conversion of the individual figures. The calculation of the standard deviation is done on the conversions, then we return to the original figures.

Results

These are arranged in Tables II, III, IV and V. They may be summarized thus:

In the liver

- The concentrations of the NAD and NADP cofactors were changed neither by gavage nor by intraperitoneal injection of citrate.
- The concentration of NADH which fell very significantly after i.p. injection of citrate was not significantly changed by gavage (Table IV).
- Two hours after gavage of the citrate mixture, a very significant decrease of NADPH was observed; no effect on this cofactor was visible 1 hour after gavage, no more than 30 minutes after i.p. injection.
- The following substrates increased after oral administration of citrate:
  - lactate, dihydroxyacetone-phosphate, oxaloacetate, malate (all very significantly) with a tendency of the last two to decrease after two hours;
  - α-glycerophosphate and acetoacetate (both very significantly);
  - i.p. injectionof citrate acted in the same way, but all of the changes were very significant.
- The concentration of β-hydroxybutyrate fell significantly one hour after gavage or after i.p. injection of citrate; two hours after gavage, it returned to normal.
- The concentration of glutamate did not significantly increase under the influence of gavage of the citrate mixture, although it increased very significantly after i.p. injection of citrate.
- The concentrations of pyruvate and α-ketoglutarate were not modified by either of the treatments.
- The level of urea was very significantly increased by gavage and i.p. injection of citrate.

Table II. Hepatic levels of lactate (LAC), β-hydroxybutyrate (βHOB), malate (MAL), α-glycerophosphate (α GP), and glutamate (GLU), expressed in μm/g of fresh weight. M ± Sm. (In parentheses, the number of rats.)

	LAC	βHOB	MAL	α GP	GLU
Control	0,541±0,0647 (5)	3,03±0,261 (6)	0,185±0,0122 (6)	0,71±0,103 (6)	2,22±0,166 (6)
Citrate i.p. 1 g/kg - 30 min	3,81 ±0,326 (6)	1,28±0,261 (6)	1,65 ±0,248 (6)	1,20±0,103 (6)	3,52±0,166 (6)
Citrate per os 5 g/kg - 1 hr	1,72 ±0,293 (6)	1,70±0,261 (6)	0,492±0,0819 (6)	0,93±0,103 (6)	2,67±0,166 (6)
Citrate per os 5 g/kg - 2 hr	1,82 ±0,293 (6)	2,97±0,261 (6)	0,379±0,0793 (6)	1.05±0,103 (6)	2,59±0,166 (6)

Table III. Hepatic levels of pyruvate (PYR), acetoacetate (ACAC), oxaloacetate (OAA), dihydroxyacetone-phosphate (DHAP), α-ketoglutarate (α KG), expressed in nanomoles/g of fresh weight. M ± Sm. (In parentheses, the number of rats.)

	PYR	ACAC	OAA	DHAP	α KG
Control	64±14,0 (7)	92,1±12,0 (6)	18,5±4,04 (5)	24,0±5,08 (6)	36,0± 9,53 (5)
Citrate i.p. 1 g/kg - 30 min	105±21,9 (9)	151 ±11,0 (6)	44,6±4,04 (5)	45,3±5,08 (6)	53 ±11,5 (6)
Citrate per os 5 g/kg - 1 hr	80±15,3 (9)	136 ± 8,38(6)	39,9±4,04 (5)	48,2±5,08 (6)	41,6± 8,72 (6)
Citrate per os 5 g/kg - 2 hr	62±10,6 (8)	213 ±37,1 (6)	31,2±4.04 (5)	46,4±5,08 (6)	22,9±4,98 (5)

Table IV. Hepatic levels of cofactors: NADH, NAD, NADPH and NADP, expressed in nanomoles/g of fresh weight. M ± Sm. (In parentheses, the number of rats.)

	NADH	NAD	NADPH	NADP
Control	256±14,7 (10)	417±20,1 (10)	359±19,2 (8)	63,7±7,04 (6)
Citrate i.p. 1 g/kg - 30 min	160±14,7 (10)	409±20,1 (10)	404±19,2 (8)	73,3±7,04 (6)
Citrate per os 5 g/kg - 1 hr	224±14,7 (10)	388±20,1 (10)	344±19,2 (8)	64,4±7,04 (6)
Citrate per os 5 g/kg - 2 hr	281±14,7 (10)	422±20,1 (10)	293±19,2 (8)	72,9±7,04 (6)

Table V. Hepatic concentrations of urea, uremia and glycemia. M ± Sm. (In parentheses, the number of rats.)

	Hepatic urea in µm/g	Uremia in g/l	Glycemia in g/l
Control	4,64±0,352 (5)	0,16	1,011±0,0538 (6)
Citrate i.p. 1 g/kg - 30 min	6,90±0,352 (5)	0,23	1,52 ±0,167 (6)
Citrate per os 5 g/kg - 1 hr	5,71±0,352 (5)	0,19	1,412±0,0971 (6)
Citrate per os 5 g/kg - 2 hr	7,96±0,352 (5)	0,25	1,78 ±0,173 (6)

In blood

Uremia and glycemia increased after either treatment.

Discussion

1 - The β-hydroxybutyrate/acetoacetate ratio decreased significantly after oral administration of the citrate mixture, very significantly after i.p. injection of citrate (Table VI). The work of Krebs (14) and Lehninger (15) showed that this ratio is an index of the intramitochondrial NADH/NAD ratio. The fall in the intramitochondrial NAD/NADH ratio can only be interpreted as a more oxidized state of the redox pair, NAD/NADH, as compared to the normal. According to Chance and Williams (5 bis), this oxidation is encountered during the utilization of the substrate provided that the amount of phosphate acceptor remains high (ADP); in other words, that all the ADP is not transformed into ATP. When the substrate is at an elevated concentration, as is the case here, the mitochondria are in state no. 3, "active", the phosphorylating oxidation being intense. This slows down in proportion to the disappearance of the substrate, but if the ADP remains at a high concentration, NADH continually decreases toward zero (state no. 2 of Chance and Williams). The total transformation of ADP into ATP brings about the total reduction of the NAD and the return to normal of the NADH/NAD ratio: this is state no. 4 "of rest", if the substrate is at a high concentration, state no. 1 if the substrate has been used up. From the preceding, we may interpret the fall in the intramitochondrial NADH/NAD ratio as indicative of an increase in activity of the Krebs cycle.

Citrate administered orally thus increases the activity of the Krebs cycle as does the i.p. administration of citrate.

The lactate/pyruvate ratio increased after both of the treatments (in the case of gavage, the dispersion of the pyruvate concentration prevents the increase in this ratio from being significant). This ratio is an index of the cytoplasmic NADH/NAD ratio. The cytoplasmic NADH/NAD ratio thus increases under our experimental conditions; the environment becomes more reducing.

The total NADH/NAD ratio (corresponding to the direct determinations which we make of these two cofactors) falls following i.p. injection of citrate, but it is no different after gavage than it is in the controls.

Table VI. Ratio of reduced forms to oxidized forms (*). M ± Sm. (In parentheses, the number of rats.)

	NADH/NAD	LAC/PYR	βHOB/ACAC	α GP/DHAP	GLU/α KG
Control	0,609±0,0506 (10)	10,3± 3,94 (5)	31,0±6,55 (5)	28,5±2,74 (5)	68±19,1 (5)
Citrate i.p. 1 g/kg - 30 min	43 ±12,3 (6)	43 ±12,3 (6)	7,8±1,69 (6)	25,6±6,15 (5)	72±17,6 (6)
Citrate per os 5 g/kg - 1 hr	0,583±0,0593 (10)	21,6± 5,02 (6)	11,4±2,31 (6)	19,8±4,77 (6)	70±14,1 (6)
Citrate per os 5 g/kg - 2 hr	0,671±0,0771 (10)	27,1±11,5 (5)	15,0±2,32 (6)	22,0±4,08	145±44,5 (4)

(*) Calculated from the individual values after normalization of the variation.

2 - The lowering of the total of the concentrations of β-hydroxybutyrate and acetoacetate which is very significant after gavage of i.p. injection, although the acetoacetate concentration increases, indicates that in both cases citrate exerts an anti-ketogenic effect. This is undoubtedly the result of the activation by the citrate (demonstrated by Waite and Wakil (26), of the carboxylation of the acetyl-CoA into malonyl-CoA, the first step in the biosynthesis of fatty acids (22). This anti-ketogenic effect indicated by the fall in the total of the concentrations of these two ketonic substances, does not appear to be lasting, since the level of β-hydroxybutyrate rises two hours after gastric intubation of the citrate mixture. But other factors make us think that it is nothing of the sort because the concentrations of citrate and oxalate remain higher than normal, and it is known that these substances facilitate the recovery of the waste products of the acetoacetate by the peripheral tissues (7). Further, it is known that so long as there is a sufficient quantity of oxaloacetate, the acetyl-coenzyme A is condensed with the oxaloacetate to form citrate (12) which is burned in the Krebs cycle.

3 - The oral administration, like the i.p. injection of citrate, activates the Krebs cycle, we have said. The ATP thus formed is necessary to the activity of the splitting enzyme of citrate which reduces this substrate to acetyl-CoA and oxaloacetate (3, 20). The proof of the activation of this enzyme (16) is the increase of the concentrations of malate and oxalacetate which the activity of the Krebs cycle cannot account for (the increase in the activity of the Krebs cycle cannot be verified by the increase in the concentrations of the intermediaries).

The oxaloacetate thus formed may, by the effect of the carboxykinase of phosphoenol-pyruvate, yield phosphoenol-pyruvate (8), the first step of gluconeogenesis which is facilitated by the reducing nature of the cytoplasmic milieu (elevation of the lactate-pyruvate ratio). Moreover, it is known that citrate inhibits the reaction catalyzed by phospho-fructokinase, the key enzyme in glycolysis (21). The very significant elevation in glycemia is the result of the inhibition of glycolysis by the citrate and the stimulation of gluconeogenesis by the oxaloacetate. Finally, under our conditions in which glycolysis is inhibited (25), the increase of the concentrations of dihydroxyacetone phosphate and of α-glycerophosphate is additional proof of gluconeogenesis (24).

4 - Lipogenesis and the synthesis of triglycerides necessitates the simultaneous contribution of acetyl-CoA, NADPH and α-glycerophosphate (27). The formation of malonyl-CoA is, in our opinion, activated by the citrate. The NADPH is formed either by the action of the cytoplasmic dehydrogenase of the isocitrate or perhaps by the action of the malic enzyme. The pyruvate formed by the action of the malic enzyme (exactly like that formed from phosphoenol pyruvate, in other words, oxaloacetate) is soon transformed into lactate. There again, the raising of the lactate/pyruvate ratio, in other words, the reducing nature of the cytoplasm, facilitates the biosynthesis of fatty acids (4).

An increase in the concentration of α-glycerophosphate was observed. All the conditions were thus present for the stimulation of lipogenesis (29).

5 - The glutamate/ α-ketoglutarate ratio was not significantly changed. The concentration of oxaloacetate was increased. At the same time as oxaloacetate appeared as a substrate of gluconeogenesis, it could by a transamination reaction:

$$OAA + GLU \longrightarrow \alpha\ KG + ASP,$$

bring about the formation of aspartate, in which intervention is necessary for the formation from citrulline, of arginosuccinate, the precursor of urea (10). The increase in the concentration of urea in the liver is the result of it; there results from it an elevation in uremia. It was found concurrently in the case of intraperitoneal injection, corpulence of the bladder, an indication of polyuria.

In conclusion, the variations in the concentrations of various cofactors and substrates after oral administration of a citrate mixture had the following results: increase in the activity of the Krebs cycle, anti-ketogenic effect and inhibition of glycolysis; there was also stimulation of gluconeogenesis, lipogenesis and ureogenesis.

Citrate would thus have an effect under different physiopathological conditions, especially when there is production of ketones (13): low carbohyrate,high fat, high protein diets, violent muscular exertion, anesthesia, sugar diabetes (diabetes mellitus) and ketosis.

Summary

The authors administered citrate to male Wistar Rats of roughly 130 g fasting for 18 hours, either 1 g/kg by intraperitoneal injection or 5 g/kg by mouth. They were sacrificed at the same time as controls, i.e. 30 minutes after injection or 1 or 2 hours after oral administration.

The concentration of various substrates and cofactors was determined in the liver by enzyme analysis.

The concentration of the following substances rose as a result of treatment: lactate, dihydroxyacetone phosphate, oxalocetate, malate, alpha-glycerophosphate, acetoacetate and urea. The concentration of beta-hydroxybutyrate fell. NAD, NADP, pyruvate, and alphaketoglutarate remained unchanged. NADH fell and glutamate rose after injection but not after oral ingestion.

These variations are discussed and compared with other experiments carried out with various compounds. Finally examination of the concentration ratios shows that citrate accelerates Krebs' cycle, has an antiketogenic effect and inhibits glycolysis. It seems to be able to stimulate neoglucogenesis, lipogenesis and ureogenesis.

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ÉTUDE COMPARÉE DES PERTURBATIONS MÉTABOLIQUES PROVOQUÉES DANS LE FOIE DU RAT PAR ADMINISTRATION « PER OS » ET INJECTION INTRA-PÉRITONÉALE DE CITRATE

P. BARON, G. GRIFFATON et R. LOWY (*) (**)

LES nombreux travaux des dix dernières années soulignent le rôle important du citrate comme régulateur du métabolisme énergétique (2) dans différentes conditions physiologiques (1, 23).

Le citrate intervient, en effet, aussi bien dans les systèmes de la synthèse des acides gras (18, 17, 11) que dans ceux de la glycolyse (20, 19, 6) et de la néoglucogénèse (28).

Il nous a donc paru intéressant de déterminer les effets comparés de deux modes d'administration du citrate : injection intrapéritonéale de 1 g/kg de citrate de sodium à pH : 7,2 et ingestion *per os* de 5 g/kg d'un mélange d'acide citrique et d'un sel de citrate monosodique et monopotassique (***).

Dans ce but, nous avons étudié l'effet du citrate sur les grandes fonctions biochimiques cellulaires : lipogénèse, cétogénèse, néoglucogénèse, oxydations et glycolyse.

Nous apprécions la cétogénèse par la somme des concentrations de β-hydroxybutyrate et acétoacétate. La concentration de l'α-glycérophosphate est un facteur régulateur de la lipogénèse. Nous apprécions l'état global d'oxydo-réduction et le potentiel de synthèse de la cellule par le rapport NADH/NAD total, tandis que le rapport lactate/pyruvate reflète le rapport NADH/NAD cytoplasmique et que le rapport β-hydroxybutyrate/acétoacétate est un indice du rapport NADH/NAD intramitochondrial, donc de l'activité du cycle de Krebs. La détermination de l'α-cétoglutarate, du glutamate, du malate, de l'oxaloacétate donne des indications complémentaires sur le fonctionnement du cycle de Krebs, celle de l'oxaloacétate permettant en plus d'apprécier la néoglucogénèse.

Nous avons donc pour ce travail déterminé dans le foie les concentrations des cofacteurs sous forme oxydée (NAD, NADP) et sous forme réduite (NADH, NADPH) et des substrats suivants : lactate, pyruvate, β-hydroxybutarate, acétoacétate, malate, oxaloacétate, citrate, α-cétoglutarate, glutamate, α-glycéro-phosphate et dihydroxyacétone-phosphate. Cette série de déterminations a été complétée par le dosage de la concentration de l'urée dans le foie, de l'urémie et de la glycémie.

PROTOCOLE EXPERIMENTAL

A la suite d'expériences préliminaires dans lesquelles le mélange citrate était apporté à la dose de 2,5 g/kg per os et sur lesquelles il était difficile de conclure, nous avons repris notre expérimentation avec des doses plus fortes. Au-delà de 5 g/kg, il nous est apparu que, la solution devenant plus pâteuse, le tubage gastrique devenait plus difficile. Nous avons donc testé les effets du citrate administré per os à la dose de 5 g/kg. L'expérimentation a été réalisée sur des rats mâles Wistar, de 125-135 g, à jeun depuis 18 heures.

Par des dosages de citrate dans la cavité gastrique et dans le sang, nous avons déterminé, par rapport à la quantité du mélange citrate administrée, la quantité de citrate retrouvée et, par différence, la quantité de citrate métabolisée (tableau I).

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(***) Composition du mélange : acide citrique, 1,30 g ; citrate mono-K, 1,76 g ; citrate mono-Na, 1,845 g ; triméthoxybenzène, 0,20 g ; jaune soleil, 1 mg ; cyclamate Na, 115 mg ; saccharinate Na, 11 mg ; essence d'orange, 9,2 mg ; essence de mandarine, 2,5 mg.

Nous sommes reconnaissants aux Laboratoires Lafon de nous avoir aimablement fourni ce mélange.

TABLEAU I. — Pourcentage de citrate retrouvé et métabolisé après injection intrapéritonéale et après gavage de citrate. Teneur hépatique en citrate dans les mêmes conditions. (Entre parenthèses, nombre de rats).

	(1) % de mélange sel de citrate et d'acide citrique retrouvé	(2) % de mélange sel de citrate et d'acide citrique métabolisé	(3) Teneur hépatique en citrate en nmol/g ; M ± S_M
(4) Témoin	—	—	88 ± 10,0 (4)
5 Citrate i.p. 1 g/kg rat tué 30 mn après	5	95	1 542 ± 50,9 (4)
6 Citrate per os 5 g/kg rat tué 1 h après	63	37	257 ± 18,4 (4)
7 Citrate per os 5 g/kg rat tué 2 h après	53	47	218 ± 41,2 (3)
8 Citrate per os 5 g/kg rat tué 3 h après	36	64	181 ± 20,5 (3)

Nous avons fait ces déterminations, en même temps que celle de la concentration hépatique du citrate, sur 4 groupes de rats :

— des rats témoins ne recevant aucun traitement. Une étude préalable a montré que les éléments autres que l'ion citrate, administrés lors de l'injection i.p. ou du gavage, sont sans effet ;

— des rats soumis à une injection i.p. de 1 g/kg de citrate et sacrifiés 30 minutes après l'injection. Ce lot nous permet de comparer les effets du citrate administré par gavage à ceux, rapides et massifs, de l'apport de citrate par voie i.p. ;

— des rats soumis au mélange citrate (5 g/kg) par tubage gastrique et sacrifiés, un lot une heure après le tubage, le second 1 heure après et le troisième 2 heures après.

Ce tableau montre qu'il n'est pas bon d'attendre 3 heures, car, bien que la quantité métabolisée augmente avec le temps, il faut avoir des concentrations suffisamment différentes de celles du témoin pour apprécier les effets. C'est pourquoi, dans la suite du travail, nous avons effectué les déterminations des substrats et des cofacteurs (voir introduction) sur les 4 premiers lots de rats.

Pour ces dosages, nous avons utilisé des modifications personnelles des méthodes enzymatiques publiées par Bergmeyer (5).

Le foie, prélevé rapidement et lavé dans KCl isotonique, est coupé en deux morceaux dont l'un est plongé immédiatement dans l'azote liquide. Les substrats sont dosés après extraction perchlorique à l'Ultra-Turrax et élimination de l'acide en excès. Sur l'autre morceau de foie, nous déterminons les formes réduites et oxydées des dinucléotides, après extraction du tissu dans un tampon phosphate à ébullition.

Il n'a été tenu compte ni des contaminations par le sang, ni de l'espace intracellulaire vrai.

Nous exprimons les résultats en nanomoles ou en micromoles par gramme de foie frais. Les valeurs obtenues ont été soumises à l'analyse de variance. Lorsque les variances des différents groupes à comparer forment une population normalement distribuée (on le vérifie au moyen de tests appropriés) l'analyse de variance aboutit au calcul d'un écart-type commun à tous ces groupes. Le degré de signification des différences entre groupes a été évalué au moyen du test F.

Dans le cas du calcul des écarts-type sur les moyennes des rapports, il est nécessaire de normaliser la variance par une transformation angulaire des données individuelles. Le calcul de l'écart-type est fait sur ces transformées, puis on revient aux données originelles.

RESULTATS

Ils sont rassemblés dans les tableau II, III, IV et V. On peut les résumer ainsi.

Dans le foie :

— Les concentrations des cofacteurs NAD et NADP ne sont modifiées ni par le gavage, ni par l'injection intrapéritonéale de citrate.

— La concentration de NADH qui baisse très significativement après l'injection i.p. de citrate n'est pas modifiée significativement par le gavage (Tableau IV).

— 2 heures après gavage au mélange citrate, on observe une diminution très significative du NADPH ; aucun effet sur ce cofacteur n'est visible 1 heure après gavage, non plus que 30 minutes après injection intrapéritonéale.

— Les substrats suivants augmentent après administration de citrate *per os* :

- lactate, dihydroxyacétone-phosphate, oxaloacétate, malate (tous très significativement) avec tendance à la diminution au bout de 2 heures pour les deux derniers ;
- α-glycérophosphate et acétoacétate (tous deux significativement) ;
- l'injection i.p. de citrate agit dans le même sens mais les variations sont toutes très significatives.

— La concentration du β-hydroxybutyrate baisse très significativement 1 heure après gavage ou après injection i.p. de citrate ; 2 heures après gavage, elle revient à la normale.

TABLEAU II. — Teneurs hépatiques en lactate (LAC), β-hydroxybutyrate (βHOB), en malate (MAL), en α-glycérophosphate (αGP), en glutamate (GLU), exprimées en µm/g de poids frais. M ± s_M. (Entre parenthèses, nombre de rats).

	LAC	βHOB	MAL	αGP	GLU
(1) Témoin	0,541 ± 0,0647 (5)	3,03 ± 0,261 (6)	0,185 ± 0,0122 (6)	0,71 ± 0,103 (6)	2,22 ± 0,166 (6)
Citrate i.p. 1 g/kg - 30 mn.	3,81 ± 0,326 (6)	1,28 ± 0,261 (6)	1,65 ± 0,248 (6)	1,20 ± 0,103 (6)	3,52 ± 0,166 (6)
Citrate per os 5 g/kg - 1 h.	1,72 ± 0,293 (6)	1,70 ± 0,261 (6)	0,492 ± 0,0819 (6)	0,93 ± 0,103 (6)	2,67 ± 0,166 (6)
Citrate per os 5 g/kg - 2 h.	1,82 ± 0,293 (6)	2,97 ± 0,261 (6)	0,379 ± 0,0793 (6)	1,05 ± 0,103 (6)	2,59 ± 0,166 (6)

TABLEAU III. — Teneurs hépatiques en pyruvate (PYR), acétoacétate (ACAC), oxaloacétate (OAA), dihydroxyacétone-phosphate (DHAP), α-cétoglutarate (αKG) exprimées en nanom/g de poids frais. M ± s_M. (Entre parenthèses, nombre de rats).

	PYR	ACAC	OAA	DHAP	αKG
Témoin	64 ± 14,0 (7)	92,1 ± 12,0 (6)	18,5 ± 4,04 (5)	24,0 ± 5,08 (6)	36,0 ± 9,53 (5)
Citrate i.p. 1 g/kg - 30 mn.	105 ± 21,9 (9)	151 ± 11,0 (6)	44,6 ± 4,04 (5)	45,3 ± 5,08 (6)	53 ± 11,5 (6)
Citrate per os 5 g/kg - 1 h.	80 ± 15,3 (9)	136 ± 8,38 (6)	39,9 ± 4,04 (5)	48,2 ± 5,08 (6)	41,6 ± 8,72 (6)
Citrate per os 5 g/kg - 2 h.	62 ± 10,6 (8)	213 ± 37,1 (6)	31,2 ± 4,04 (5)	46,4 ± 5,08 (6)	22,9 ± 4,98 (5)

TABLEAU IV. — Teneurs hépatiques en cofacteurs : NADH, NAD, NADPH et NADP, exprimées en nanom/g de poids frais. M ± s_M. (Entre parenthèses, nombre de rats).

	NADH	NAD	NADPH	NADP
Témoin	256 ± 14,7 (10)	417 ± 20,1 (10)	359 ± 19,2 (8)	63,7 ± 7,04 (6)
Citrate i.p. 1 g/kg - 30 mn.	160 ± 14,7 (10)	409 ± 20,1 (10)	404 ± 19,2 (8)	73,3 ± 7,04 (6)
Citrate per os 5 g/kg - 1 h.	224 ± 14,7 (10)	388 ± 20,1 (10)	344 ± 19,2 (8)	64,4 ± 7,04 (6)
Citrate per os 5 g/kg - 2 h.	281 ± 14,7 (10)	422 ± 20,1 (10)	293 ± 19,2 (8)	72,9 ± 7,04 (6)

TABLEAU V. — Concentrations hépatiques de l'urée, urémie et glycémie. M ± s_M. (Entre parenthèses, nombre de rats).

	Urée hépatique en µm/g	Urémie en g/l	Glycémie en g/l
Témoin	4,64 ± 0,352 (5)	0,16	1,011 ± 0,0538 (6)
Citrate i.p. 1 g/kg - 30 mn.	6,90 ± 0,352 (5)	0,23	1,52 ± 0,167 (6)
Citrate per os 5 g/kg - 1 h.	5,71 ± 0,352 (5)	0,19	1,412 ± 0,0971 (6)
Citrate per os 5 g/kg - 2 h.	7,96 ± 0,352 (5)	0,25	1,78 ± 0,173 (6)

— La concentration du glutamate n'augmente pas significativement sous l'influence du gavage au mélange citrate alors qu'elle augmente très significativement après une injection i.p. de citrate.

— Les concentrations du pyruvate et de l'α-cétoglutarate ne sont modifiées par aucun des traitements.

— La teneur en urée est très significativement augmentée par le gavage et l'injection i.p. de citrate.

Dans le sang :

L'urémie et la glycémie augmentent après l'un ou l'autre des traitements.

DISCUSSION

1. — Le rapport β-hydroxybutyrate/acétoacétate diminue significativement après le gavage au mélange citrate, très significativement après injection i.p. de citrate (Tableau VI). Les travaux de Krebs (14) et de Lehninger (15) ont montré que ce rapport est un indice du rapport NADH/NAD intramitochondrial. La baisse du rapport NADH/NAD intramitochondrial ne peut que traduire un état plus oxydé du couple redox NAD/NADH par rapport à la normale. Selon Chance et Williams (5 bis) cet état d'oxydation se rencontre au cours de l'utilisation du substrat, pourvu que la teneur en accepteur de phosphate (ADP) reste élevée, c'est-à-dire tant que tout l'ADP n'est pas transformé en ATP. Lorsque le substrat est à concentration élevée, comme c'est le cas ici, les mitochondries sont dans l'état n° 3 « actif », les oxydations phosphorylantes sont intenses. Celles-ci se ralentissent au fur et à mesure que le substrat disparaît, mais si l'ADP reste à concentration élevée, le NADH diminue toujours et tend vers zéro (état n° 2 de Chance et Williams). La transformation complète de l'ADP en ATP entraîne la réduction complète du NAD et le retour à la normale du rapport NADH/NAD : c'est l'état n° 4 « de repos », si le substrat est à concentration élevée, l'état n° 1, si le substrat a été consommé. De ce qui précède, nous pouvons interpréter l'abaissement

TABLEAU VI. — Rapport des formes réduites aux formes oxydées (*). M ± s_M. (Entre parenthèses, nombre de rats).

	NADH/NAD	LAC/PYR	βHOB/ACAC	αGP/DHAP	GLU/αKG
Contrôle Témoin	0,609 ± 0,0506 (10)	10,3 ± 3,94 (5)	31,0 ± 6,55 (5)	28,5 ± 2,74 (5)	68 ± 19,1 (5)
Citrate i.p. 1 g/kg - 30 mn.	43 ± 12,3 (6)	43 ± 12,3 (6)	7,8 ± 1,69 (6)	25,6 ± 6,15 (5)	72 ± 17,6 (6)
Citrate per os 5 g/kg - 1 h.	0,583 ± 0,0593 (10)	21,6 ± 5,02 (6)	11,4 ± 2,31 (6)	19,8 ± 4,77 (6)	70 ± 14,1 (6)
Citrate per os 5 g/kg - 2 h.	0,671 ± 0,0771 (10)	27,1 ± 11,5 (5)	15,0 ± 2,32 (6)	22,0 ± 4,08	145 ± 44,5 (4)

(*) Calculé sur les valeurs individuelles après normalisation de la variance.

du rapport NADH/NAD intramitochondrial comme traduisant une augmentation de l'activité du cycle de Krebs.

Le citrate *per os* accroît donc l'activité du cycle de Krebs comme le fait l'administration i.p. de citrate.

Le rapport lactate/pyruvate augmente après l'un ou l'autre des traitements (dans le cas du gavage, la dispersion des concentrations du pyruvate empêche que l'augmentation de ce rapport soit significative). Ce rapport est un indice du rapport NADH/NAD cytoplasmique (9). Le rapport NADH/NAD cytoplasmique s'élève donc dans nos conditions expérimentales ; le milieu devient plus réducteur.

Le rapport NADH/NAD total (correspondant aux déterminations directes que nous faisons de ces deux cofacteurs) baisse à la suite de l'injection i.p. de citrate, mais n'est pas différent après le gavage de ce qu'il est chez le témoin.

2. — La baisse de la somme des concentrations de β-hydroxybutyrate et acétoacétate qui est très significative après le gavage ou injection i.p., bien que la concentration de l'acétoacétate augmente, indique que le citrate exerce dans les deux cas un effet anticétogénique. Ceci est sans doute la conséquence de l'activation par le citrate, démontré par Waite et Wakil (26), de la carboxylation de l'acétyl-CoA en malonyl-CoA, première étape de la biosynthèse des acides gras (22). Cet effet anticétogénique indiqué par la baisse de la somme des concentrations de ces deux corps cétoniques n'apparaît pas durable, puisque la teneur en β-hydroxybutyrate s'élève deux heures après le tubage gastrique au mélange citrate. Mais d'autres éléments permettent de penser qu'il n'en est rien, car les concentrations de citrate et d'oxaloacétate restent supérieures à la normale et l'on sait que ces substrats facilitent le captage de l'acétoacétate par les tissus périphériques (7). D'ailleurs, on sait que, tant que l'oxaloacétate est à concentration suffisante, l'acétyl-coenzyme A est condensé avec l'oxaloacétate pour former du citrate (12) qui est brûlé dans le cycle de Krebs.

3. — L'administration *per os* comme l'injection i.p. de citrate, active, nous l'avons dit, le cycle de Krebs. L'ATP ainsi formé est nécessaire à l'activité de l'enzyme de coupure de citrate qui dégrade ce substrat en acétyl-CoA et oxaloacétate (3, 20). La preuve de l'activation de cet enzyme (16) est l'augmentation des concentrations de malate et d'oxaloacétate dont l'accroissement d'activité du cycle de Krebs ne peut rendre compte (l'augmentation d'activité du cycle de Krebs ne peut se constater par l'augmentation des concentrations des intermédiaires).

L'oxaloacétate ainsi formé peut par l'action de la carboxykinase du phosphoénol-pyruvate donner le phosphoénol-pyruvate (8), première étape de la néoglucogénèse qui est facilitée par le caractère réducteur du milieu cytoplasmique (élévation du rapport lactate/pyruvate). On sait, par ailleurs, que le citrate inhibe la réaction catalysée par la phospho-fructokinase, enzyme-clé de la glycolyse (21). L'élévation très significative de la glycémie est la conséquence de l'inhibition de la glycolyse par le citrate et de la stimulation de la néoglucogénèse à partir de l'oxaloacétate. Enfin, dans nos conditions où la glycolyse est inhibée (25), l'augmentation des concentrations de dihydroxyacétonephosphate et d'α-glycérophosphate est une preuve supplémentaire de la néoglucogénèse (24).

4. — La lipogénèse et la synthèse des triglycérides exigent l'apport simultané d'acyl-CoA, de NADPH et d'α-glycérophosphate (27). La formation de malonyl-CoA est, nous l'avons vu, activée par le citrate. Le NADPH se forme soit par l'action de la déshydrogénase cytoplasmique de l'isocitrate, soit peut-être par l'action de l'enzyme malique. Le pyruvate formé par l'action de l'enzyme malique (tout comme celui qui se forme à partir du phosphoénol-pyruvate, c'est-à-dire de l'oxaloacétate) à partir du malate, dont la concentration est augmentée, est aussitôt transformé en lactate. Là encore l'élévation du rapport lactate/pyruvate, c'est-à-dire le caractère réducteur du cytoplasme, facilite la biosynthèse des acides gras (4).

L'augmentation de la concentration de l'α-glycérophosphate a été observée. Toutes les conditions sont donc réunies pour stimuler la lipogénèse (29).

5. — Le rapport glutamate/α-cétoglutarate n'est pas significativement modifié. La concentration de l'oxaloacétate est augmentée. Dans le même temps que l'oxaloacétate apparaît comme un substrat de la néoglucogénèse, il peut, par la réaction de transamination :

$$OAA + GLU \rightarrow \alpha KG + ASP,$$

réaliser la formation d'aspartate, dont l'intervention est nécessaire à la formation, à partir de la citrulline, de l'arginosuccinate, précurseur de l'urée (10). L'augmentation de la concentration de l'urée du foie en est la conséquence : il en résulte une élévation de l'urémie. On constate parallèlement, dans le cas de l'injection intrapéritonéale, une réplétion de la vessie, indice d'une polyurie.

En conclusion, les variations des concentrations des cofacteurs et des substrats, après administration *per os* du mélange citrate, ont les conséquences suivantes : accroissement de l'activité du cycle de Krebs, effet anticétogénique et inhibition de la glycolyse ; il y a aussi stimulation de la néoglucogénèse, de la lipogénèse et de l'uréogénèse.

Le citrate aurait donc un effet dans différentes conditions physiopathologiques surtout lorsqu'il y a production de corps cétoniques (13) : régimes hypoglucidiques, hypergras, hyperprotéiques, effort musculaire violent, anesthésie, diabète sucré et cétoses.

RÉSUMÉ

Des rats mâles Wistar de 130 g environ, à jeun depuis 18 heures, reçoivent une certaine dose de citrate (soit 1 g/kg par injection i.p. soit 5 g/kg per os). Ils sont sacrifiés en même temps que les témoins, soit 30 minutes après l'injection, soit 1 et 2 heures après l'ingestion.

On détermine dans leur foie, par analyse enzymatique, la concentration de divers subtrats et cofacteurs.

La concentration des composés suivants s'élève sous l'effet des traitements : lactate, dihydroxyacétone-phosphate, oxaloacétate, malate, α-glycerophosphate et acétoacétate, urée. Celle du β-hydroxybutyrate s'abaisse. Les concentration en NAD, NADP, pyruvate et α-cétoglutarate ne sont pas modifiées. La concentration du NADH s'abaisse et celle du glutamate s'élève après l'injection, mais non après l'ingestion.

La discussion de ces variations, la comparaison avec d'autres expériences effectuées avec divers composés, enfin la considération des rapports des concentrations, montrent que le citrate augmente l'activité du cycle de Krebs, a un effet anticétogénique et inhibe la glycolyse. Il paraît capable de stimuler la néoglucogénèse, la lipogénèse et l'uréogénèse.

SUMMARY

The authors administered citrate to male Wistar Rats of roughly 130 g. fasting for 18 hours, either 1 g/kg by intraperitoneal injection or 5 g/kg by mouth. They were sacrificed at the same time as controls, i.e. 30 minutes after injection or 1 or 2 hours after oral administration.

The concentration of various substrates and cofactors was determined in the liver by enzyme analysis.

The concentration of the following substances rose as a result of treatment : lactate, dihydroxyacetone phosphate, oxalocetate, malate, alpha-glycerophosphate, acetoacetate and urea. The concentration of beta-hydroxybutyrate fell. NAD, NADP, pyruvate, and alphaketoglutarate remained unchanged. NADH fell and glutamate rose after injection but not after oral ingestion.

These variations are discussed and compared with other experiments carried out with various compounds. Finally examination of the concentration ratios shows that citrate accelerates Krebs' cycle, has an antiketogenic effect and inhibits glycolysis. It seems to be able to stimulate neoglucogenesis, lipogenesis and ureogenesis.

RESUMEN

Unos machos de Rata Wistar de aproximadamente 130 g, en ayunas desde 18 horas, reciben cierta dosis de citrato (sea 1 g/kg por inyección intraperitoneal, sea 5 g/kg per os). Se les sacrifica al mismo tiempo que los controles, sea 30 minutos después de la inyección, sea 1 y 2 horas después de la ingestión.

Es determinada en su hígado, por análisis enzimática, la concentración de diversos substratos y cofactores.

La concentración de los compuestos siguientes se eleva bajo el efecto de los tratamientos : lactato, dihidroxiacetonafosfato, oxalocetato, malato, α-glicerofosfato y acetoacetato, urea. La del β-hidroxi butarato se abaja. Las concentraciones en NAD, NADP, piruvato y α-cetoglutarato no están modificadas. La concentración del NADH se rebaja y la del glutamato se eleva después de inyección, pero no después de ingestión.

La discusión de estas variaciones, la comparación con otros experimentos efectuados con diversos compuestos, y por fin la consideración de las relaciones de las concentraciones, muestran que el citrato aumenta la actividad del ciclo de Krebs, surte efecto anticetogénico e inhibe la glicólisis. Parece capaz de estimular la neoglucogénesis, la lipogénesis y la ureogénesis.

ZUSAMMENFASSUNG

Seit 18 Stunden nüchterne Wistar-Rattenmännchen von etwa 130 g erhalten eine bestimmte Zitratdosis (entweder 1 g/kg per injectionem i.p. oder 5 g/kg per os). Sie werden entweder 30 Minuten nach der Injektion oder 1 und 2 Stunden nach der Ingestion zugleich mit den Kontrolltieren geopfert.

Man bestimmt in ihrer Leber durch Enzymanalyse die Konzentration verschiedener Substrate und Kofaktoren.

Die Konzentration folgender Verbindungen steigt unter dem Einfluss der Behandlungen: Laktat, Dihydroxyazetonphosphat, Oxalocetat, Malat, α-Glyzerophosphat und Azetoazetat, Harnstoff. Die des β-Hydroxybutyrates sinkt. Die NAD-, NADP-, Pyruvat- und α-Cetoglutaratkonzentrationen ändern sich nicht. Die NADH-Konzentration sinkt und die Glutamatkonzentration steigt nacht der Injektion, aber nicht nach der Ingestion.

Die Erörterung dieser Variationen, der Vergleich mit anderen, an verschiedenen Verbindungen angestellten Versuchen und schliesslich die Betrachtung der Beziehungen der Konzentrationen zeigen, dass das Zitrat die Aktivität des Krebszyklus erhöht, einen anticetogenen Einfluss hat und die Glykolyse hemmt. Es scheint die Neoglukogenese, die Lipogenese und die Urogenese anregen zu können.

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THE EFFECT OF CITRIC ACID ON THE TERATOGENIC ACTION OF TRYPAN BLUE[1]

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Trypan blue has been shown to be teratogenic when injected into the subgerminal cavity or yolk sac of the developing chicken egg (1,2,3,4). The most common abnormality observed after such treatment is rumplessness. The mechanism of action of this dye is not known, although, it has been proposed that it acts primarily on the mesoderm (3,4).

Landauer has obtained evidence which shows that certain teratogens are active during chick development by interfering with metabolic processes. He has demonstrated that the teratogenic effects of some compounds can be prevented, or lessened, by supplying the embryos with compounds that play a role in metabolism (5).

The present study was undertaken to determine if a metabolic compound could modify the teratogenic effects of trypan blue in the chicken egg. Citric acid was selected for testing because of its effectiveness in preventing induced rumplessness.

Materials and Methods

Fertile eggs of the White Leghorn chicken were obtained from

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three different commercial sources. The eggs were injected into the yolk sac at 36 hours incubation: Hamburger-Hamilton stages 9-11 (6). Trypan blue[2] was prepared as a 0.1% saline solution and 0.1ml was injected. This dose has been shown to be teratogenic in the developing hen's egg (7). Citric acid[3] was prepared in saline so that the injected volume of 0.1ml contained 25 mg of citric acid. Twenty-five milligrams of citric acid has been used successfully by Landauer in preventing insulin induced rumplessness (8). Citric acid was injected first followed immediately by trypan blue. Control eggs were either injected with saline or incubated without injection. The embryos were recovered on the 10th day of incubation, fixed, weighed and examined for gross malformations.

Results

Table 1 summarizes the results of this experiment. It is evident that when citric acid is injected in conjunction with trypan blue there can be a marked reduction in the teratogenic effects of the dye. In the eggs from flocks one and two there was a reduction in mortality from 41% to 27% and from 56% to 30% respectively. The incidence of malformations was reduced by more than one-half in treated eggs from each flock. Statistical analysis by the chi-square method revealed these reductions to be significant (see table for P values). Identical experiments with eggs of the third flock resulted in an increase in mortality and did not reduce the numbers malformed to a significant degree.

Discussion and Conclusions

The results of this experiment show that it is possible to

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[2]Matheson, Coleman & Bell Co. Norwood, Ohio

[3]Nutritional Biochemicals Corp,. Cleveland, Ohio

TABLE 1

The Effect of Citric Acid and Trypan Blue on Chick Development when Injected into Eggs of Three Different Flocks at the 36th Hour of Incubation[1]

	Total treated	Percentage mortality	Percentage malformed survivors	Percentage all eggs affected
Flock 1				
Untreated controls	231	5.6	2.3	7,8
Saline controls	135	17.0	5.4	21.4
Citric acid	182	17.0	1.3	18.1
Trypan blue	226	41.2	30.3	58.8
Trypan blue & Citric acid	462	27.3 (P= 0.001)[2]	10.1 (P= 0.001)	34.6 (P= 0.001)
Flock 2				
Untreated controls	134	11.9	3.4	14.9
Saline controls	141	12.0	2.4	14.2
Citric acid	77	5.2	1.4	6.5
Trypan blue	94	56.5	56.2	71.2
Trypan blue & Citric acid	153	29.8 (P= 0.001)	19.3 (P= 0.001)	43.5 (P= 0.001)
Flock 3				
Untreated controls	117	2.6	0.8	3.4
Saline controls	121	11.5	1.9	13.2
Citric acid	91	8.8	6.0	14.3
Trypan blue	156	49.4	45.6	72.4
Trypan blue & Citric acid	134	61.9	31.4 (P= 0.10)	73.9

[1]Dosage: 0.1mg trypan blue; 25mg citric acid.

[2]Probability determined by chi-square method.

interfere with the teratogenic action of trypan blue in the chicken egg by simultaneous treatment with citric acid. Landauer has shown conclusively that supplementation with certain chemicals of metabolic importance to the chick embryo can protect against the action of certain teratogens. The effectiveness of this protection varies with the teratogenic agent, the supplemental agent and the embryonic period (5). One of the agents Landauer found most effective in preventing insulin induced sumplessness was pyruvic acid. I have used pyruvic acid in combination with trypan blue in 104 eggs of flock two, but no protection was afforded the embryos of these eggs. Citric acid is an effective protecting agent in eggs of the same flock. The action of trypan blue is apparently restricted to the first 96 hours of chick development with a peak in activity at 36 hours (7). It has been shown that the peak in the incidence of rumplessness induced by insulin follows treatment at 31 hours of development and that pyruvic acid and citric acid are effective as protective agents if given within 3 hours of the injection of insulin (8,9). Thus there is similarity in both embryonic period and type of malformation produced between eggs treated with insulin and eggs treated with trypan blue. Further experiments will be necessary to determine the nature of the interaction between trypan blue and citric acid. It is possible that some inactivation of the trypan blue molecule takes place at the injection site, e.g., due to the formation of a complex between dye and acid, or due to a local change in pH.

The use of eggs from three different flocks of White Leghorn chickens points up the importance of genetic variation in teratogenesis. In the present experiment protection against the embryotoxic and teratogenic effects of trypan blue by citric acid was

most marked in eggs from two of the three flocks. In the third flock the protection was lacking, in fact, there was a synergistic action in the lethal effect. All factors of treatment and incubation were the same for each flock, the only apparent difference is genetic. Landauer (10) has shown significant differences in response to the teratogenic effects of boric acid by the eggs of different mothers within the same flock. Also suggestive of genetic variation among the flocks was the observation that malformed embryos from flock 2 had, in addition to rumplessness, many more types of defects than embryos from either flock 1 or 3. In the latter two flocks rumplessness was most often the only defect exhibited by the embryo.

Landauer and Rhoades (8) have proposed that insulin induced rumplessness is due to a disruption in carbohydrate metabolism. It is too early to postulate the same for trypan blue induced rumplessness although the results of this first series of experiments would be in agreement with such a postulate.

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STUDIES ON THE ABSORPTION OF CITRIC ACID IN THE GASTROINTESTINAL TRACT OF SUCKLING RATS

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As a contribution to the question of acidulated milk food for human babies, studies were set up on the behavior of citric acid in the gastro-intestinal tract of rat sucklings. When giving 400 mg. of citric acid per kilogram of body weight, marked differences occur from adult rats. The acid transport from the stomach to the small intestine is considerably delayed, and its stay in the small intestine is prolonged. The citrate content in the blood of rat sucklings which is normally twice as high as in adults, increases after peroral administration of the acid more slowly and to a smaller extent, thus indicating a delayed absorption. The activities of aconitate hydratase and isocitrate dehydrogenase in the small intestine mucosa are slightly reduced so that a delayed metabolism of citric acid may be expected compared with adults. The results are discussed with respect to the response of human babies to acidulated milk food.

Some time ago, we reported on the location, extent and rate of absorption of citric acid, and its enzymic conversion in the gastrointestinal tract of adult rats (1). This study showed that when 64 mg. citric acid is given orally to rats weighing about 150g (427 mg) kg body weight), almost 25% of the acid is absorbed by the small intestine within 100 min. and supplied to the liver with portal vein blood, whereas 65% is metabolized (mainly through the citric acid cycle) during the course of absorption in the mucosa. As a continuation of these studies, we have examined the course of citric acid absorption in suckling rats. This study was prompted by the renewed very lively discussion during the past few years on the pros and cons of acidulated milk feeding of human infants.

Droese et al. (2-8) studied, during the first 3 months of life, especially the behavior of infants fed milk mixtures with or without the addition of lactic acid. From the results, it was concluded that an increased supply of organic acids or acid valencies exceeds the functional capacity of the liver and kidneys

* We wish to thank Miss Ch. Fritz, Miss R. Urbat and Mrs. H. Kohler for their conscientious experimental collaboration.

in some of the infants (1/4 to 1/3 of the examined test subjects); this leads to a metabolic acidosis with a lowering of the alkali reserve and an increase of chlorine and residual nitrogen in the blood. The absorption and retention of nitrogen are not improved, but even frequently show a change for the worse. These findings are particularly strongly expressed with 4/5 mixtures rich in milk. After addition of citric acid to 1/2 and 2/3 milk mixtures (1 citrette = 0.38 g citric acid per 100 ml whole milk), the authors found a slight increase in the amount of organic acids present in urine, and further a definite decrease of citric acid and ketonebodies. Whether the latter findings constitute an advantage or a drawback for a healthy infant is still a matter which must be clarified, although Droese and Stolley (6, 8) report that citric acid causes a slight deterioration, particularly in young infants, of the fat and base utilization and leads to an increased spitting and vomiting and thin stools.

In contrast to these findings by Droese are data reported by Donath (12), according to which thousands of infants fed acidulated milk thrived quite well. According to results obtained by Zeisel et al. (9, 10), milk diets acidulated with citric acid do not cause in 1-6 month-old infants, any significant changes in different metabolic data, such as citrate content, pH value, CO_2 partial pressure, and standard bicarbonate in blood. Simon et al. (11) point out that additions of acid to milk preparations constitute a safety factor with regard to a possible nitrate reduction caused by microbes. It is worth mentioning that, according to results obtained by Baumgartner and Ketz (13), young albino rats fed a diet containing added citric and lactic acid exhibit a significantly greater increase in body weight than control animals. If we compare the amounts of citric acid supplied with drinking amounts equal to 1/6 of the body weight, then we obtain the values listed in Table 1; this amount is divided into 5 meals (feeding periods) per day. According to Table 1, the infants receive, when fed 2/3 cow's milk, 2.7 times as much, when fed Citrosan, 6.2 times as much, and when fed citrettes + 2/3 cow's milk, 7 times as much citric acid as compared to infants fed on mother's milk. The citric acid content of rat milk is equal to 200 mg/500 g milk. Therefore, a suckling rat of 10 g body weight, when drinking an amount of 2.3 g milk per 24 hours, takes up about 92 mg citric acid/day/kg body weight. This value is of the same order of magnitude as that of the human infant (compare Table 1).

Table 1. Citric Acid Content of Different Infant Milk Diets.

Type of Milk	Citric Acid[3], mg	Citric Acid Supply (Intake), in mg/day/kg Body Weight
Women's Milk	500	83
2/3 Cow's Milk	1334	222
Citrosan[1]	3076	510
Citrettes + 2/3 Cow's Milk[2]	3446	575

[1] VEB Condensed Milk Factory Stendal and Genthin; 100 ml milk diet contain 15 g Citrosan powder.

[2] Chemical Works Joh. A. Benckiser GmbH, Ludwigshafen/Rhein; 1 citrette per each 100 ml whole milk.

[3] Personal studies.

The purpose of our studies is to provide a further contribution, in view of the discrepancies reported in the literature, as to whether there are any differences in the metabolism and absorption of orally administered citric acid, using suckling rats as a model, in comparison to previously tested adult rats.

Methods

Studies were carried out on 10-day old Wistar rats, weighing about 10 g, of both sexes (Rehbrucke breeding strain), which were weaned from their mothers 2 hours before the start of tests.

Determinations of the Extent and Rate of Absorption of Citric Acid

In the citric acid loading tests, the animals received 4.0 mg citric acid in 0.1 ml physiological saline by gastric intubation; this dose is equal to 400 mg/kg BW and thus is somewhat lower than the daily supply (intake) of a human infant fed a milk diet containing citric acid (Table 1). The entire amount is given at once in a single dose, in order that the results of measurements should lie beyond (outside) the biological variation and can be compared with our earlier results obtained with adult rats (1).

4-29 animals are killed by decapitation (control animals immediately, and animals subject to a citric acid load 30, 60, 90, 120, 150 and 180 minutes after

acid administration); to the collected blood, heparin is added as usual to prevent coagulation. From the extracted gastrointestinal tract, the stomach, small intestine (including the cecum) and colon are separately weighed, and for investigation (in view of the partially very small citric acid content) 4-6 organs (sometimes more) are combined and placed in 5 ml of a 20% trichloroacetic acid solution; to 5 ml blood, 5 ml trichloroacetic acid is also added. Further pretreatment and the determination of citric acid are carried out as described previously (1). Urine is collected by puncture from the more or less full bladder; the citrate content is determined in collective samples taken from about 15 animals, and the pH is measured with a microglass electrode in individual or collective samples.

Activities of Aconitate Hydratase (EC 4.2.1.3) and Isocitrate Dehydrogenase (EC 1.1.1.42) in the Small Intestine Mucosa

Extraction of the small intestine mucosa and determination of the enzyme activities was done as described in our previous report (1); the protein content of the enzyme solutions is determined according to Folin-Ciocalteu (14).

Results

Absorption and Metabolism of Citric Acid in the Gastrointestinal Tract

The weights of the stomach, small intestine and colon (with their contents), and the citric acid content of these organs and in the blood and urine of suckling rats not subject to a citric acid load are given in Table 2. Formulas (1) and (2) are used for statistical evaluation; when the amount of citric acid present is very small, several organs are combined into one sample, and formulas (2) are then used.

$$s = \pm\sqrt{\frac{\Sigma x_i^2 - \frac{\Sigma (x_i)^2}{N}}{N-1}}; \qquad \bar{x} = \frac{\Sigma x_i}{N}; \qquad v\,[\%] = \frac{s \cdot 100}{\bar{x}} \qquad (1)$$

$$s = \pm\sqrt{\frac{\Sigma z_i x_i^2 \frac{(\Sigma z_i x_i)^2}{N}}{N-1}}; \qquad \bar{x}_g = \frac{\Sigma z_i x_i}{N}; \qquad v\,[\%] = \frac{s \cdot 100}{\bar{x}_g} \qquad (2)$$

where s = standard deviation; x_i = single value; N = number of single values; x = arithmetic mean; z_i = number of animals for one sample; $\bar{x}_g$ = weighed arithmetic mean; v = variability coefficient.

Table 2. Weight and Citric Acid Content of Different Organs and Body Fluids of Suckling Rats.

Organ	Weight				Citric Acid								
	No. of Animals (N)	$\bar{x}$ [g]	s [g]	v [%]	No. of Animals (N)	No. of Samples (P)	No. of Animals for one Sample (z_i)	$\bar{x}_g$ [µg/g Tissue]	s [µg/g Tissue]	v [%]	$\bar{x}_g$ [µg/ Animal]	s [µg/ Animal	v [%]
Stomach	108	0,382	±0,207	±54,2	108	8	6-29	43,8	±16,8	±38,3	15,0	±2,8	±18,7
Small intestine	108	0,481	±0,087	±18,1	108	8	6-29	55,1	± 8,6	±15,6	26,0	±3,9	±15,0
Colon	66	0,096	±0,025	±26,0	66	3	18-29	67,8	± 4,6	± 6,8	6,46	±1,50	±23,2
Blood	41	About 0,3 g/animal			41	5	7- 9	55,0	± 1,7	± 3,1	-	-	-
Urine	42	About 0.07 ml/animal			42	3	13-15	24,4[1]	± 1,6[1]	± 6,6	-	-	-

[1] µg/ml

Listed in Table 3 are those amounts of citric acid ("surplus value") which are obtained, after oral administration of 4000 micrograms, in the digestive organs of one animal after subtracting values of animals not subjected to a citric acid load (Table 2). The still rudimentary cecum was included in the small intestine. For methodical reasons, a determination of citric acid in the portalvein blood of suckling rats was not carried out, thus eliminating the possibility of differentiating between absorbed citric acid and citric acid metabolized in the mucosa.

In some cases the pH value and citric acid content of urine were determined. No differences could be detected in tests without and with citric acid loading (30 min); in both cases, the pH values lie between 5.1 and 6.8 (30 samples) and the citrate content between 22 and 30 µg/ml (3 samples).

Activity of Aconitate Hydratase and Isocitrate Dehydrogenase of the Small Intestine Mucosa

The determined specific activities are listed in Table 4. Values in the cecum and colon could not be determined since the amount of mucosa was too small; for comparison purposes, the corresponding findings on adult rats (1) are given in Table 4.

Discussion

If we compare the results obtained with suckling and adult rats (1), similar relations can be seen from a qualitative standpoint. During a test period of 3 hours following oral loading, citric acid is continuously removed from the stomach; it is absorbed in the small intestine and metabolized by aconitate hydratase and isocitrate dehydrogenase present in the mucosa, and thus does not reach the colon. The citrate content of the blood undergoes a temporary increase during absorption.

On the other hand, quantitative relations are clearly different in the two rat age groups tested. A balance of the distribution of citric acid during the absorption time period investigated in suckling rats and adult animals is shown in Figure 1. It is apparent that in suckling rats the readiness (tendency) to undergo metabolism and absorption during the first 90 minutes is very low; in contrast, in adult animals, about 85% of the administered citric acid has been absorbed or metabolized during the same time period. After these initial 90

Table 3. Surplus Value of Citric Acid in the Stomach, Small Intestine, Colon and Blood of Suckling Rats After Oral Administration of 4000 Micrograms Citric Acid.

Organ		Absorption Time						
		0	30	60	90	120	150	180
Stomach								
No. of Animals (N)		-	61	76	30	49	30	30
No. of Samples (P)		-	12	14	5	6	5	5
No. of Animals for One Sample (z_1)		-	4-6	5-6	6	6-19	6	6
Surplus value of citric acid	$\bar{x}_g$ [µg/animal]	4000	3077	2559	2000	1111	546	580
	s [µg/animal]	-	±358	±513	±508	±116	±185	± 31
	v [%]	-	± 12	± 20	± 25	± 10	± 34	± 5
Small Intestine								
No. of Animals (N)		-	56	76	30	49	30	30
No. of Samples (P)		-	11	14	5	6	5	5
No. of Animals for One Sample (z_1)		-	4-6	5-6	6	6-19	6	6
Surplus value of citric acid	$\bar{x}_g$ [µg/animal]	0	852	1169	1768	911	1148	242
	s [µg/animal]	-	±278	±164	±280	±169	±109	±129
	v [%]	-	± 34	± 14	± 16	± 18	± 10	± 53

Table 3. Surplus Value of Citric Acid in the Stomach, Small Intestine, Colon and Blood of Suckling Rats After Oral Administration of 4000 Micrograms Citric Acid (Cont'd)

Organ		Absorption Time 0	30	60	90	120	150	180
Colon								
No. of Animals (N)		-	30	30	60	30	30	30
No. of Samples (P)		-	5	5	10	5	5	5
No. of Animals for One Sample (z_i)		-	6	6	6	6	6	6
Surplus value of citric acid	$\bar{x}_g$ [µg/animal]	0	-2.1	1.4	-2.4	-0.6	2.1	-1.8
	s [µg/animal]	-	±0.6	±2.3	±0.9	±0.4	±3.0	±0.5
	v [%]	-	±29	±164	±38	±67	±143	±28
Blood								
No. of Animals (N)		-	64	76	30	30	30	30
No. of Samples (P)		-	10	11	5	5	5	5
No. of Animals for One Sample (z_i)		-	4-8	6-8	6	6	6	6
Surplus value of citric acid	$\bar{x}_g$ [µg/g]	0	4.3	10.9	2.3	25.0	0.4	-2.2
	s [µg/g]	-	+7.2	+11.8	±4.2	±20.2	±2.1	±6.2
	v [%]	-	±168	±108	±183	±81	±552	±282

Table 4. Aconitate Hydratase and Isocitrate Dehydrogenase in the Small Intestine Mucosa of Rats

		Suckling Rats	Adult Rats
No. of Animals (N)		145	9
No. of Samples (P)		6	2
No. of Animals for One Sample (z_i)		17-30	4-5
Amount of Mucosa per Rat	$\bar{x}_g$ [g]	0.106	2.62
	s [g]	± 0.050	± 0.22
	v [%]	±47.2	± 8.4
Protein per 1 g Mucosa	$\bar{x}_g$ [mg]	67.2	43.3
	s [mg]	±15.8	± 3.5
	v [%]	±23.5	± 8.1
Specific Activity[1]			
Aconitate Hydratase	$\bar{x}_g$	0.010	0.020
	s	± 0.004	± 0.007
	v [%]	±40.0	±35.0
Isocitrate Dehydrogenase	$\bar{x}_g$	0.068[2]	0.094
	s	± 0.010	± 0.009
	v [%]	±14.7	± 9.6

1 - Specific activity = micromoles of substrate metabolized per min and per mg of protein at 20°C.

2 - N = 98; P = 4; z_i = 17-30.

minutes, which in suckling rats can be considered as a "lag" or adaptation period, the acid undergoes the above conversions in suckling rats at almost the same rate as was already noted at the beginning of the test in adult rats. In the infant animals, the rate at which citric acid leaves the stomach is considerably reduced; the residual percentage fraction of the administered acid is accordingly clearly increased (greater) in the stomach during the entire absorption period investigated.

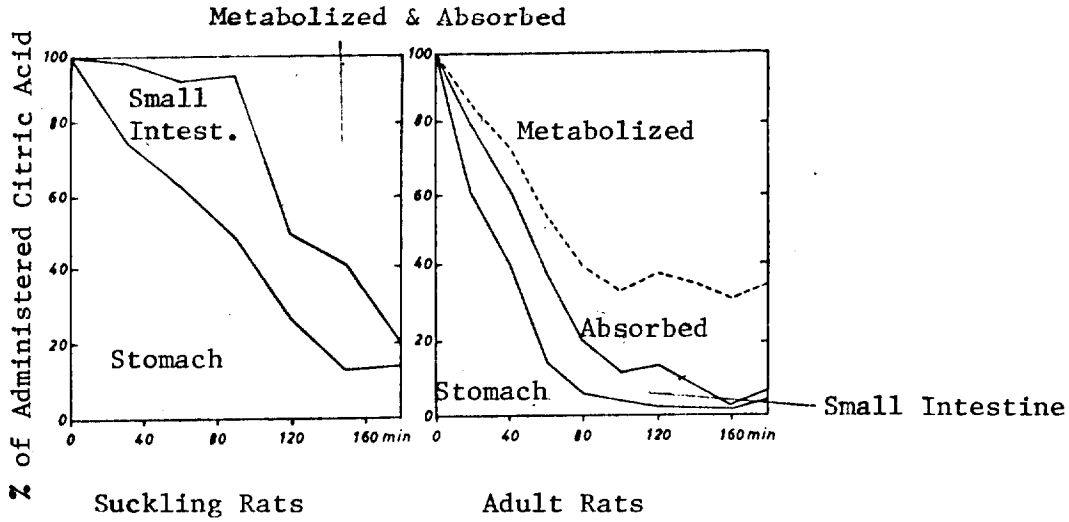

Figure 1. Distribution of Citric Acid in the Digestive Tract After Oral Administration of Citric Acid to Suckling Rats (4 mg/animal = 400 mg/kg BW = 100%) and Adult Rats (64 mg/animal = 427 mg/kg BW = 100%).

The selected citric acid loading was about the same for suckling rats (400 mg/kg BW) and adult animals (427 mg/kg BW). One must consider, however, that the percentage share, based on the body weight, of digestive organs is quite different in both age groups of rats (Table 5).

Table 5. Proportion (share) of Stomach, Small Intestine and Colon, Based on Total Body Weight, in Suckling and Adult Rats.

Organ	Suckling Rats (10 g)		Adult Rats (150 g)	
	[g]	% of body wt (KG)	[g]	% of body wt (KG)
Stomach	0.382	3.82	1.87	1.25
Small Intestine	0.481	4.81	5.74	3.82
Colon	0.0096	0.96	1.64	1.08

If the amount of citric acid administered is related to the weight of the stomach, then we obtain a citric acid loading of 10.5 mg acid per gram stomach for suckling rats, while the corresponding figure for adult rats is 34.9 mg/g stomach, i.e. it is 3.3 times greater. Figure 2 shows the absorption behavior of citric acid according to this computation basis. The considerably greater rate of decrease of this acid in the stomach of adult rats is particularly clearly expressed.

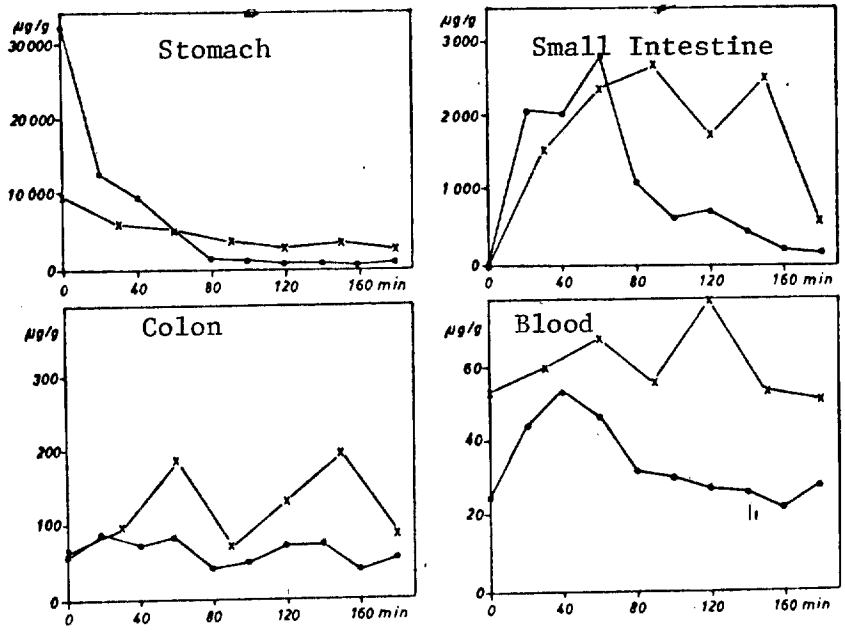

Figure 2. Citric Acid Content of Stomach, Small Intestine, Colon (Each with Its Own Contents) and Blood After Oral Administration of Citric Acid to Suckling and Adult Rats.

x——x Suckling Rats o——o Adult Rats

The increase in citric acid in the small intestine is at first of about the same order of magnitude in both groups; however, the citrate contents of adult rats tend to return to the original values at a considerably faster rate. In the colon, a certain increase of citric acid values could not be detected in both groups. The apparently higher values noted in suckling rats after 60, 120 and 150 minutes must be attributed to the accidental, particularly low weights of the colons of the

animals (compare Table 3). The citrate content of blood in suckling rats not exposed to a citric acid load is about 2 times as high as in adult rats. In the latter, however, a sharp peak is noted during the first third of the test period, but after 80-100 minutes (end of the skin absorption phase) the citrate values drop down again to their initial value. On the other hand, in suckling rats (except for fluctuations, which are always greater in this group than in adult animals), only a flat peak extending over the entire test period can be observed; the slightly protruding peak noted after 120 minutes in this case has its counterpart in the corresponding minimum in the small intestine, and must be considered as an extreme example of the biological variation. Apparently, the suckling rats originating from 4 litters, examined on one day and belonging to the groups tested 120 minutes after being subjected to a citric acid load, exhibit a particularly good (high) absorption capacity.

The very small amounts of acid absorbed and metabolized during the first 90 minutes in suckling rats (Figure 1) can be explained, in part, by the reduced (although not to a great extent) activities of aconitate hydratase and isocitrate dehydrogenase (Table 4). At the same time, a considerable lag in the absorption is also noted, as can be seen from the increased citrate values in the small intestine and in the only slowly increasing values of blood citrate.

The observations of Droese et al. (2-8) on the partially poor tolerance of citric acid in very young human infants are supported to a certain extent by our results obtained with suckling rats. The metabolism and absorption of citric acid administered orally to these suckling rats is clearly lower than in adult animals; this fact cannot be considered as a load (strain) on the metabolism in the digestive tract, which is not yet capable of functioning at a fully normal rate, but rather as a strain (load) on the blood and other organs of the intermediate metabolism, since apparently absorption is clearly slowed down. The conditions selected by us in our animal tests, namely a single administration of the daily dose without additional food, are quite extreme and unilateral as compared to those of human infants in which the daily dose is subdivided into 5 meals (feeding periods) with a milk diet. Although conclusions applicable to the human infant can be drawn from our results with definite caution and reservations, these results still provide valuable criteria for key problems that have to de clarified in regard to an acidulated infant diet.

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Untersuchungen zur Resorption von Zitronensäure im Magen-Darm-Kanal von Rattensäuglingen

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(Eingegangen am 14. 12. 1965)

Zusammenfassung

Als Beitrag zum Für und Wider einer gesäuerten Milchnahrung bei menschlichen Säuglingen werden Untersuchungen über das Verhalten der Zitronensäure im Magen-Darm-Trakt von Rattensäuglingen beschrieben. Bei einer Belastung mit 400 mg Zitronensäure/1 kg Körpergewicht treten gegenüber erwachsenen Ratten Unterschiede auf. Der Säuretransport vom Magen in den Dünndarm ist beträchtlich verlangsamt, die Verweildauer im Dünndarm verlängert. Der von Natur aus beim Rattensäugling etwa doppelt so hohe Zitratgehalt im Blut gegenüber erwachsenen Tieren steigt nach peroraler Zufuhr der Säure deutlich langsamer und geringer an, was auf verzögerte Resorption schließen läßt. Die Aktivitäten der Akonitat-hydratase und Isozitrat-dehydrogenase in der Dünndarmmukosa sind bei den Tiersäuglingen leicht erniedrigt, so daß gegenüber erwachsenen Tieren mit einem verzögerten metabolischen Umsatz der Zitronensäure zu rechnen ist. Die Ergebnisse werden im Hinblick auf das Verhalten des menschlichen Säuglings gegenüber gesäuerter Milchnahrung diskutiert.

Vor einiger Zeit berichteten wir über Ort, Umfang und Geschwindigkeit der Resorption von Zitronensäure sowie über ihre enzymatische Umsetzung im Magen-Darm-Trakt erwachsener Ratten [1]. Danach werden 64 mg peroral an etwa 150 g schwere Tiere verabfolgte Zitronensäure (427 mg/kg Körpergewicht (KG), innerhalb von 100 min im Dünndarm zu fast 25% resorbiert und mit dem Portalvenenblut der Leber zugeführt, während etwa 65% im Verlauf der Resorption in der Mukosa der Metabolisierung — im wesentlichen über den Zitronensäurezyklus — unterliegen. In Fortsetzung dieser Untersuchungen haben wir den Resorptionsverlauf der Zitronensäure bei Rattensäuglingen verfolgt. Veranlassung hierzu war die in den letzten Jahren sehr rege wiederaufgegriffene Diskussion um das Für und Wider einer gesäuerten Milchnahrung für menschliche Säuglinge.

[1] Für gewissenhafte experimentelle Mitarbeit danken wir Fräulein Ch. Fritz, Fräulein R. Urban und Frau H. Koehler.

DROESE et al. [2—8] untersuchten im ersten Trimenon insbesondere das Verhalten von Säuglingen, die mit Milchmischungen ohne bzw. mit Milchsäurezusatz ernährt wurden. Aus den Ergebnissen wurde gefolgert, daß das erhöhte Angebot an organischen Säuren bzw. sauren Valenzen bei einem Teil der Säuglinge ($^1/_4$—$^1/_3$ der untersuchten Probanden) die Leistungsfähigkeit von Leber und Nieren übersteigt; dies führt zu metabolischer Azidose mit Erniedrigung der Alkalireserve und zur Erhöhung von Chlor und Reststickstoff im Blut. Resorption und Retention von Stickstoff werden nicht verbessert, sondern häufig sogar verschlechtert. Diese Befunde sind bei milchreichen $^4/_5$-Mischungen besonders ausgeprägt. Nach Zitronensäurezusatz zur $^1/_2$- und $^2/_3$-Milchmischung (1 Citrette = 0,38 g Zitronensäure auf 100 ml Vollmilch) finden die Autoren im Harn eine geringe Zunahme an organischen Säuren, ferner eine deutliche Abnahme der Zitronensäure und der Ketonkörper. Ob die zuletzt genannten Befunde für den gesunden Säugling vor- oder nachteilig sind, steht noch dahin, doch geben DROESE und STOLLEY [6, 8] an, daß die Zitronensäure insbesondere bei jungen Säuglingen die Fett- und Basenausnutzung leicht verschlechtert und zum vermehrten Spucken und Erbrechen sowie zu dünnen Stühlen führt.

Diesen Befunden von DROESE stehen Angaben von DONATH [12] gegenüber, wonach Tausende von Säuglingen mit angesäuerter Milch gut gediehen. Nach Ergebnissen von ZEISEL et al. [9, 10] rufen mit Zitronensäure gesäuerte Milchnahrungen bei 1—6 Monate alten Säuglingen signifikante Änderungen bei verschiedenen Stoffwechseldaten (Zitratgehalt, *p*H-Wert, CO_2-Partialdruck und Standard-Bikarbonat im Blut) nicht hervor. SIMON et al. [11] weisen darauf hin, daß Säurezusätze zu Milchzubereitungen einen Sicherheitsfaktor gegenüber einer möglichen, mikrobiell bedingten Nitratreduktion darstellen. Nicht unerwähnt sei, daß nach Ergebnissen von BAUMGÄRTNER und KEIZ [13] mit Zitronen- bzw. Milchsäurezusatz ernährte junge Albinoratten gegenüber Kontrolltieren eine signifikant höhere Zunahme des KG aufwiesen.

Vergleicht man die Mengen an Zitronensäure, die bei Trinkmengen von $^1/_6$ des Körpergewichtes zugeführt werden, so ergeben sich die in Tabelle 1 angegebenen Werte;

Tabelle 1 Tabelle 1

Zitronensäuregehalt von verschiedenen Säuglings-Milchnahrungen

(1) Milchart	(2) Zitronensäure [3] mg%	(3) Zitronensäurezufuhr mg je 1 Tag und 1 kg KG
(4) Frauenmilch	500	83
(5) $^2/_3$-Kuhmilch	1334	222
Citrosan[1]	3076	510
(6) Citretten = $^2/_3$-Kuhmilch[2]	3446	575

[1] VEB Dauermilchwerke Stendal und Genthin; 100 ml Milchnahrung enthalten 15 g Citrosan-Pulver.

[2] Chemische Fabrik Joh. A. Benckiser GmbH, Ludwigshafen Rhein; 1 Citrette je 100 ml Vollmilch.

[3] Eigene Untersuchungen

diese Menge ist auf 5 Mahlzeiten am Tag verteilt. Nach Tabelle 1 erhalten also die Säuglinge bei Ernährung mit $^2/_3$-Kuhmilch die 2,7fache, mit Citrosan die 6,2fache, mit Citretten = $^2/_3$-Kuhmilch die 7,0fache Menge an Zitronensäure gegenüber der Ernährung mit Muttermilch. Der Zitronensäuregehalt von Rattenmilch liegt bei 200 mg/500 g Milch. Danach nimmt ein Rattensäugling von 10 g KG bei einer Trinkmenge von 2,3 g Milch 24 Std. etwa 92 mg Zitronensäure je 1 Tag und 1 kg KG auf. Dieser Wert liegt in gleicher Größenordnung wie beim menschlichen Säugling (vgl. Tab. 1).

Tabelle 2

Gewicht und Zitronensäuregehalt verschiedener Organe und Körperflüssigkeiten von Rattensäuglingen

Organ	Gewicht: Anzahl der Tiere (N)	Gewicht: $\bar{x}$ [g]	Gewicht: s [g]	Gewicht: v [%]	Gewicht: Anzahl der Tiere (N)	Zitronensäure: Anzahl der Proben (P)	Zitronensäure: Anzahl der Tiere für 1 Probe (z_i)	Zitronensäure: $\bar{x}$ [µg/g Gewebe]	Zitronensäure: s [µg/g Gewebe]	Zitronensäure: v [%]	Zitronensäure: $\bar{x}_g$ [µg/Tier]	Zitronensäure: s [µg/Tier]	Zitronensäure: v [%]
(3) Magen	108	0,382	±0,207	±54,2	108	8	6–29	43,8	±16,8	±38,3	15,0	±2,8	±18,7
(4) Dünndarm	108	0,481	±0,087	±18,1	108	8	6–29	55,1	± 8,6	±15,6	26,0	±3,9	±15,0
(5) Dickdarm	66	0,096	±0,025	±26,0	66	3	18–29	67,8	± 4,6	± 6,8	6,46	±1,50	±23,2
(6) Blut	41	etwa 0,3 g/Tier			41	5	7– 9	55,0	± 1,7	± 3,1	–	–	–
(7) Harn	42	etwa 0,07 ml/Tier			42	3	13–15	24,4[1]	± 1,6[1]	± 6,6	–	–	–

[1] µg/ml

Ziel unserer Untersuchungen ist es, im Hinblick auf die im Schrifttum zutage tretenden Diskrepanzen weitere Beiträge zu liefern, ob während Umsatz und Resorption von peroral verabfolgter Zitronensäure bei als Modell herangezogenen Rattensäuglingen Unterschiede gegenüber den früher getesteten erwachsenen Ratten bestehen.

Methodik

Die Untersuchungen wurden an 10 Tage alten, etwa 10 g schweren Wistarratten beiderlei Geschlechts (Zucht Rehbrücke) durchgeführt; sie wurden 2 Std. vor Versuchsbeginn von der Mutter abgesetzt.

Bestimmungen über Umfang und Geschwindigkeit der Resorption von Zitronensäure

Bei den Zitronensäurebelastungen erhalten die Tiere 4,0 mg Zitronensäure in 0,1 ml physiologischer Kochsalzlösung per Schlundsonde; das sind 400 mg/1 kg KG und liegt somit etwas unter der Tageszufuhr eines menschlichen Säuglings bei Ernährung mit Zitronensäure-Milchnahrung (Tab. 1). Die gesamte Menge wird auf einmal verabfolgt, damit die Meßresultate außerhalb der biologischen Schwankungsbreite liegen und mit unseren früheren Ergebnissen an erwachsenen Ratten [1] vergleichbar sind.

4–29 Tiere (Kontrolltiere sofort, Tiere mit Belastung jeweils 30, 60, 90, 120, 150 bzw. 180 min nach der Zitronensäure-Applikation) werden durch Dekapitation getötet; dem aufgefangenen Blut wird zur Verhinderung der Gerinnung wie üblich Heparin zugesetzt. Vom herauspräparierten Magen-Darm-Trakt werden Magen, Dünndarm (einschließlich Blinddarm) sowie Dickdarm einzeln gewogen, zur Untersuchung aber — wegen des z. T. sehr geringen Zitronensäuregehaltes — jeweils 4–6 der Organe (in einigen Fällen auch mehr) vereinigt und in 5 ml einer 20%igen Trichloressigsäure gelegt; 0,5 ml

Blut erhalten einen Zusatz von ebenfalls 5 ml Trichloressigsäure. Die weitere Vorbehandlung sowie die Bestimmung der Zitronensäure erfolgt wie bereits beschrieben [1].
Harn wird durch Punktion aus der meist mehr oder weniger gefüllten Harnblase entnommen; man ermittelt den Zitratgehalt in Sammelproben von etwa 15 Tieren, den *p*H-Wert in Einzel- oder in Sammelproben mittels einer Mikro-Glaselektrode.

Aktivitäten von Akonitat-hydratase (EC 4.2.1.3) und Isozitrat-dehydrogenase (EC 1.1.1.42) in der Dünndarm-Mukosa

Gewinnung der Dünndarm-Mukosa und Bestimmung der Fermentaktivitäten erfolgten gemäß unserer früheren Mitteilung [1]; der Proteingehalt der Enzymlösungen wird nach FOLIN-CIOCALTEU [14] ermittelt.

Ergebnisse

Resorption und Umsatz von Zitronensäure im Magen-Darm-Kanal

Die Gewichte von Magen, Dünndarm und Dickdarm (mit Inhalt), die Zitronensäuregehalte dieser Organe sowie die von Blut und Harn unbelasteter Rattensäuglinge sind in Tabelle 2 wiedergegeben. Zur statistischen Auswertung dienen die Formeln (1) und (2); bei sehr geringer Menge an vorliegender Zitronensäure werden mehrere Organe zu 1 Probe vereinigt und dann die Formeln (2) benutzt.

$$s = \pm \sqrt{\frac{\sum x_i^2 - \frac{\sum (x_i)^2}{N}}{N-1}}; \qquad \bar{x} = \frac{\sum x_i}{N}; \qquad v\,[\%] = \frac{s \cdot 100}{\bar{x}} \tag{1}$$

$$s = \pm \sqrt{\frac{\sum z_i x_i^2 - \frac{(\sum z_i x_i)^2}{N}}{N-1}}; \qquad \bar{x}_g = \frac{\sum z_i x_i}{N}; \qquad v\,[\%] = \frac{s \cdot 100}{\bar{x}_g} \tag{2}$$

Es bedeuten:
s = Standardabweichung; x_i = Einzelwert; N = Anzahl der Einzelwerte; $\bar{x}$ = arithmetisches Mittel; z_i = Anzahl der Tiere für 1 Probe; $\bar{x}_g$ = gewogenes arithmetisches Mittel; v = Variabilitätskoeffizient

In Tabelle 3 sind diejenigen Mengen an Zitronensäure angegeben, die nach peroraler Applikation von 4000 μg in den Verdauungsorganen von jeweils 1 Tier nach Abzug der Werte ohne Belastung (Tab. 2) erhalten werden („Mehrwert"). Der noch rudimentäre Blinddarm wurde dem Dünndarm zugeordnet. Auf eine Bestimmung von Zitronensäure im Portalvenenblut der Rattensäuglinge — und damit auf eine Unterscheidungsmöglichkeit zwischen resorbierter und in der Mukosa umgesetzter Zitronensäure — wurde aus methodischen Gründen verzichtet.
In einigen Fällen werden *p*H-Wert und Zitronensäure im Harn ermittelt; Unterschiede lassen sich bei den Versuchen ohne und mit Zitronensäurebelastung (30 min) nicht erkennen; die *p*H-Werte liegen jeweils zwischen 5,1 und 6,8 (30 Proben) und die Zitratgehalte zwischen 22 und 30 μg/ml (3 Proben).

Table 3.

Tabelle 3. Mehrwert an Zitronensäure in Magen, Dünndarm, Dickdarm und Blut von Rattensäuglingen nach peroraler Applikation von 4000 µg Zitronensäure

Organ		Resorptionszeit [min] 0	30	60	90	120	150	180
→ Magen								
(1) Anzahl der Tiere (N)		—	61	76	30	49	30	30
(2) Anzahl der Proben (P)		—	12	14	5	6	5	5
(3) Anzahl der Tiere für 1 Probe (z_i)		—	4—6	5—6	6	6—19	6	6
(4) Mehrwert an Zitronensäure	$\bar{x}_g$ [µg/Tier]	4000	3077	2559	2000	1111	546	580
	s µg/Tier	—	±358	±513	±508	±116	±185	± 31
	v [%]	—	± 12	± 20	± 25	± 10	± 34	± 5
→ Dünndarm								
(1) Anzahl der Tiere (N)		—	56	76	30	49	30	30
(2) Anzahl der Proben (P)		—	11	14	5	6	5	5
(3) Anzahl der Tiere für 1 Probe (z_i)		—	4—6	5—6	6	6—19	6	6
(4) Mehrwert an Zitronensäure	$\bar{x}_g$ [µg/Tier]	0	852	1169	1768	911	1148	242
	s µg/Tier	—	±278	±164	±280	±169	±109	±129
	v [%]	—	± 34	± 14	± 16	± 18	± 10	± 53
→ Dickdarm								
(1) Anzahl der Tiere (N)		—	30	30	60	30	30	30
(2) Anzahl der Proben (P)		—	5	5	10	5	5	5
(3) Anzahl der Tiere für 1 Probe (z_i)		—	6	6	6	6	6	6
(4) Mehrwert an Zitronensäure	$\bar{x}_g$ [µg/Tier]	0	— 2,1	1,4	— 2,4	— 0,6	2,1	— 1,8
	s µg/Tier	—	± 0,6	± 2,3	± 0,9	± 0,4	± 3,0	± 0,5
	v [%]	—	± 29	±164	± 38	± 67	±143	± 28
→ Blut								
(1) Anzahl der Tiere (N)		—	64	76	30	30	30	30
(2) Anzahl der Proben (P)		—	10	11	5	5	5	5
(3) Anzahl der Tiere für 1 Probe (z_i)		—	4—8	6—8	6	6	6	6
(4) Mehrwert an Zitronensäure	$\bar{x}_g$ [µg/g]	0	4,3	10,9	2,3	25,0	0,4	— 2,2
	s [µg/g]	—	± 7,2	± 11,8	± 4,2	± 20,2	± 2,1	± 6,2
	v [%]	—	±168	±108	±183	± 81	±552	±28,2

Aktivität der Akonitat-hydratase und Isozitrat-dehydrogenase der Dünndarmmukosa

Die ermittelten spezifischen Aktivitäten sind in Tabelle 4 wiedergegeben. Auf Erfassung der Werte bei Blind- und Dickdarm mußte wegen zu geringer Menge an Mukosa verzichtet werden; zum Vergleich sind in Tabelle 4 die entsprechenden Befunde bei erwachsenen Ratten [1] angegeben.

Table 4

Tabelle 4

Akonitat-hydratase und Isozitrat-dehydrogenase in der Dünndarm-Mukosa von Ratten

		(1) Säuglinge	Erwachsene Ratten (2)
(3) Anzahl der Tiere (N)		145	9
(4) Anzahl der Proben (P)		6	2
(5) Anzahl der Tiere für 1 Probe (z_i)		17 – 30	4 – 5
(6) Mukosa-Menge je 1 Ratte	$\bar{x}_g$ [g]	0,106	2,62
	s [g]	± 0,050	± 0,22
	v [%]	±47,2	± 8,4
(7) Protein je 1 g Mukosa	$\bar{x}_g$ [mg]	67,2	43,3
	s [mg]	±15,8	± 3,5
	v [%]	±23,5	± 8,1
(8) Spezifische Aktivität[1] Akonitat-hydratase	$\bar{x}_g$	0,010	0,020
	s	± 0,004	± 0,007
	v [%]	±40,0	±35,0
(9) Isozitrat-dehydrogenase	$\bar{x}_g$	0,068[2]	0,094
	s	± 0,010	± 0,009
	v [%]	±14,7	± 9,6

[1] Spezifische Aktivität = umgesetzte µMole Substrat je 1 min und je 1 mg Protein bei 20 °C

[2] $N = 98$; $P = 4$; $z_i = 17 – 30$

Diskussion

Vergleicht man die Ergebnisse bei Rattensäuglingen mit denen bei erwachsenen Ratten [1], so manifestieren sich in qualitativer Hinsicht analoge Verhältnisse. Während einer Versuchszeit von 3 Std. nach peroraler Belastung wird die Zitronensäure kontinuierlich aus dem Magen entfernt. Sie wird im Dünndarm resorbiert bzw. durch die in der Mukosa vorhandene Akonitat-hydratase sowie Isozitrat-dehydrogenase metabolisiert und gelangt dementsprechend nicht in den Dickdarm; der Zitratgehalt des Blutes wird während der Resorption vorübergehend erhöht.

Die quantitativen Verhältnisse aber unterscheiden sich deutlich bei den 2 geprüften Altersgruppen der Ratten. Eine Bilanz über die Verteilung der

Zitronensäure im untersuchten Resorptionszeitraum bei Säuglingen und bei erwachsenen Tieren ist in Abbildung 1 wiedergegeben. Augenfällig ist, daß bei den Säuglingen die Umsatz- bzw. Resorptionsbereitschaft in den ersten

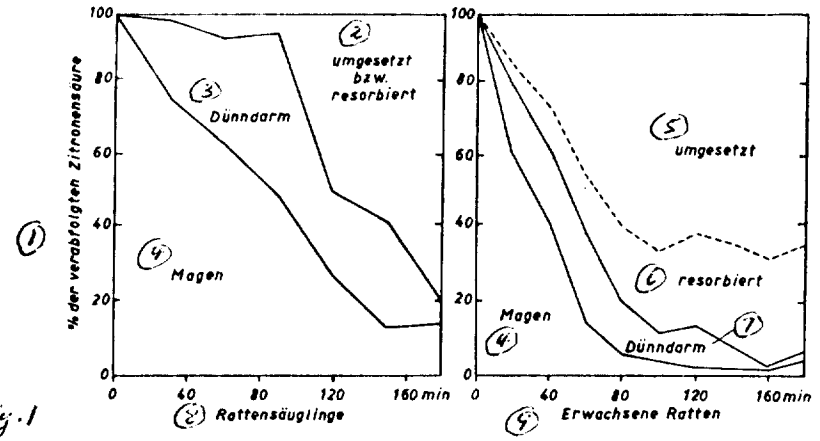

Abb. 1. Verteilung der Zitronensäure im Verdauungstrakt nach peroraler Applikation von Zitronensäure bei Rattensäuglingen (4 mg/Tier = 400 mg/kg KG = 100%) und bei erwachsenen Ratten (64 mg/Tier = 427 mg/kg KG = 100%)

90 min sehr gering ist; bei den erwachsenen Tieren sind demgegenüber zur gleichen Zeit etwa 85% der verabfolgten Zitronensäure der Resorption bzw. Metabolisierung anheimgefallen. Nach diesen 90 min, die bei den Säuglingen als eine „lag"- bzw. Adaptationsphase aufgefaßt werden können, unterliegt die Säure auch bei den Säuglingen den genannten Umsetzungen mit fast der gleichen Geschwindigkeit, wie sie bei den erwachsenen Ratten bereits zu Versuchsbeginn beobachtet worden ist. Bei den infantilen Tieren ist die Geschwindigkeit, mit der die Zitronensäure den Magen verläßt, wesentlich herabgesetzt; der verbleibende prozentuale Anteil der verabfolgten Säure ist im Magen während der gesamten untersuchten Resorptionszeit dementsprechend deutlich erhöht.

Die Belastung ist bei Säuglingen (400 mg/kg KG) und erwachsenen Tieren (427 mg/kg KG) etwa gleich groß gewählt worden. Es ist jedoch zu berücksichtigen, daß der prozentuale Anteil der Verdauungsorgane am KG bei den beiden Altersgruppen z. T. recht beträchtliche Unterschiede aufweist (Tab. 5).

Bezieht man die verabfolgte Menge Zitronensäure auf das Magengewicht, so resultiert bei den Säuglingen eine Belastung mit 10,5 mg Zitronensäure je 1 g Magen, bei den erwachsenen Tieren aber eine solche mit 34,9 mg/1 g Magen, d. h. das 3,3fache. Abbildung 2 zeigt das Resorptionsverhalten der Zitronensäure gemäß dieser Berechnungsgrundlage. Die wesentlich größere Geschwindigkeit der Abnahme an dieser Säure im Magen der erwachsenen

Tabelle 5

Tabelle 5

Anteil von Magen, Dünndarm und Dickdarm am Gesamt-Körpergewicht von Rattensäuglingen und von erwachsenen Ratten

Organ	Säuglinge (10 g)		Erwachsene Tiere (150 g)	
	[g]	[% vom KG]	g	[% vom KG]
Magen	0,382	3,82	1,87	1,25
Dünndarm	0,481	4,81	5,74	3,82
Dickdarm	0,096	0,96	1,64	1,08

Ratten kommt besonders deutlich zum Ausdruck. Die Zunahme im Dünndarm ist bei beiden Gruppen zunächst von etwa gleicher Größenordnung; die Zitratgehalte der erwachsenen Tiere streben aber wesentlich schneller

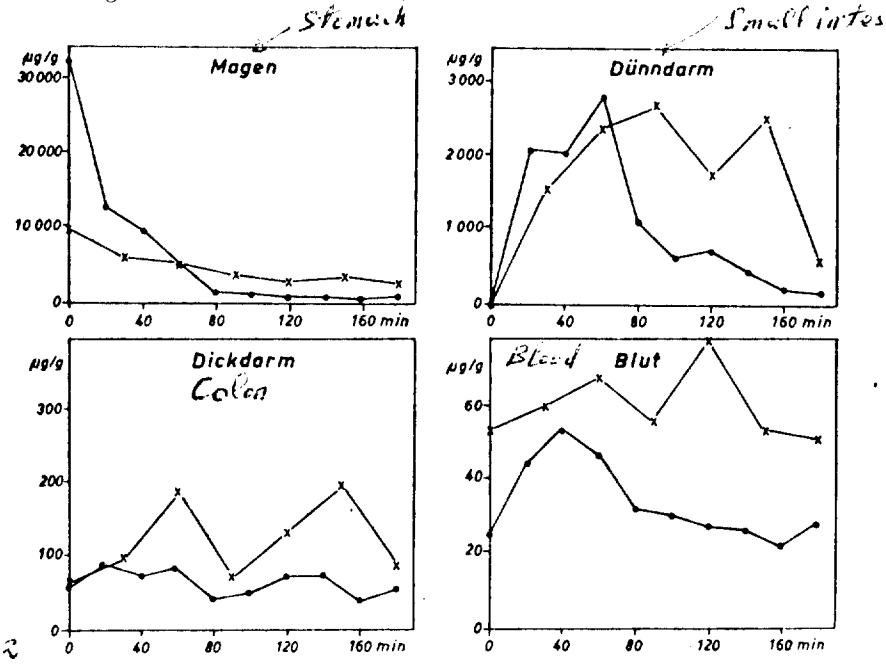

Fig. 2

Abb. 2. Gehalt an Zitronensäure im Magen, Dünndarm, Dickdarm (jeweils mit Inhalt) und Blut nach peroraler Applikation von Zitronensäure an Rattensäuglinge und erwachsene Ratten. - - × Säuglinge; ● ● Erwachsene

den Ausgangswerten zu. Im Dickdarm ist eine sichere Erhöhung der Zitronensäurewerte in beiden Fällen nicht zu erkennen. Die anscheinend höheren Werte bei den Säuglingen nach 60, 120 und 150 min sind auf zufällige, besonders niedrige Gewichte der Dickdärme der Tiere zurückzuführen (vgl. Tab. 3). Der Zitratgehalt des Blutes im unbelasteten Zustand ist bei den Säuglingen etwa doppelt so hoch wie bei den erwachsenen Tieren.

Bei letzteren tritt aber im ersten Drittel der Versuchsperiode ein scharfes Maximum auf, nach 80–100 min aber — Abschluß der Hauptresorptionsphase — sinken die Werte wieder auf den Ausgangspunkt ab. Bei den Säuglingen ist dagegen — von Schwankungen abgesehen, die in dieser Gruppe stets größer sind als bei den erwachsenen Tieren — nur ein flaches, sich über die ganze Versuchsperiode hinziehendes Maximum erkennbar; das dabei etwas herausragende Maximum nach 120 min findet seinen Gegenpol bei dem entsprechenden Minimum im Dünndarm und ist wohl als extremes Beispiel der biologischen Schwankungsbreite zu werten. Offenbar weisen die an einem Tage untersuchten, aus 4 Würfen stammenden Säuglinge der nach 120 min Belastung untersuchten Gruppen eine besonders gute Resorptionsfähigkeit auf.

Die während der ersten 90 min nur recht geringen resorbierten bzw. metabolisierten Mengen an Säure bei den Säuglingen (Abb. 1) können z. T. mit den — allerdings nicht wesentlich — verringerten Aktivitäten der Akonitathydratase und der Isozitrat-dehydrogenase erklärt werden (Tab. 4). Daneben manifestiert sich aber auch eine nicht unerhebliche Verzögerung in der Resorption, wie aus den erhöhten Zitrat-Werten im Dünndarm und den nur langsam ansteigenden Werten im Blut hervorgeht.

Die Beobachtungen von Droese et al. [2]–[8] über die z. T. schlechte Verträglichkeit von Zitronensäure bei sehr jungen menschlichen Säuglingen finden durch unsere an Rattensäuglingen erhobenen Befunde eine gewisse Stützung. Umsatz bzw. Resorption peroral an letztere verabfolgte Zitronensäure sind gegenüber erwachsenen Tieren eindeutig herabgesetzt; dies kann als eine Belastung des Stoffwechsels im noch nicht voll funktionstüchtigen Verdauungstrakt gewertet werden — weniger als eine solche des Blutes oder anderer Organe des Intermediärstoffwechsels, da offensichtlich deutlich verlangsamt resorbiert wird. Die von uns gewählten Bedingungen beim Tierversuch — einmalige Gabe der Tagesdosis ohne Beikost — sind gegenüber dem menschlichen Säugling — Tagesdosis auf 5 Mahlzeiten mit Milchnahrung verteilt — allerdings recht extrem und einseitig. Wenn auch Schlußfolgerungen aus unseren Ergebnissen auf den menschlichen Säugling nur mit Vorsicht und Zurückhaltung gezogen werden können, so vermitteln sie trotzdem wertvolle Anhaltspunkte bei der schlüssig zu klärenden Problematik der gesäuerten Säuglingsnahrung.

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Summary

U. Behnke, H.-A. Ketz and K. Täufel: Studies on the resorption of citric acid in the gastro-intestinal tract of rat sucklings

As a contribution to the question of acidulated milk food for human babies, studies were set up on the behavior of citric acid in the gastro-intestinal tract of rat sucklings. When giving 400 mg. of citric acid per kilogram of body weight, marked differences occur from adult rats. The acid transport from the stomach to the small intestine is considerably delayed, and its stay in the small intestine is prolonged. The citrate content in the blood of rat sucklings which is normally twice as high as in adults, increases after peroral administration of the acid more slowly and to a smaller extent, thus indicating a delayed absorption. The activities of aconitate hydratase and isocitrate dehydrogenase in the small intestine mucosa are slightly reduced so that a delayed metabolism of citric acid may be expected compared with adults. The results are discussed with respect to the response of human babies to acidulated milk food.

Резюме

У. Бенке, Г.-А. Кетц и К. Тойфель: Исследования о резорбции лимонной кислоты в желудочно-кишечном тракте крыс-сосунков

Как вклад к вопросу о подкисленном молочном питании грудного ребенка, авторы проводили исследования о поведении лимонной кислоты в желудочно-кишечном тракте крыс-сосунков. При введении 400 мг лимонной кислоты на 1 кг веса тела наблюдаются отличия от взрослых крыс. Транспорт кислоты от желудка в тонкий кишечник значительно замедлен, а время пребывания в тонком кишечнике повышено. Содержание цитрата в крови, которое у крыс-сосунков примерно вдвое больше, чем у взрослых животных, повышается при пероральном введении кислоты значительно медленнее, что указывает на замедление резорбции. Активности аконитат-гидратазы и изоцитрат-дегидрогеназы в мукозе тонкого кишечника слегка понижены у сосунков, так что в сравнении с взрослыми животными можно ожидать замедление метаболического обмена лимонной кислоты. Полученные данные обсуждаются в отношении реакции грудного ребенка к подкисленному молочному питанию.

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INDUCTION OF EXPERIMENTAL TETANY IN CATTLE[1]

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Burau and Stout (1965) and Stout, Brownell and Burau (1967) have found high levels of *trans*-aconitate in early season forage grasses of California. These samples were collected in connection with especially severe seasonal outbreaks of grass tetany (hypomagnesemia). The relation of *trans*-aconitate or closely related compounds to the incidence of grass tetany has been only cursorily examined. Burt and Thomas (1961) lowered plasma magnesium (Mg) by feeding sodium citrate to young calves (4 to 6 mo. of age) but not to a level associated with tetany. Plasma Mg has usually been less than 1.0 mg./100 ml. when tetany has occurred (Dozsa, 1959). Levels of *trans*-aconitate in California plants (Burau and Stout, 1965; Stout *et al.*, 1967) were higher than the level of sodium citrate used by Burt and Thomas (1961).

Studies were initiated to investigate the effect of *trans*-aconitic acid and related compounds on the Mg content of bovine plasma and its possible relationship to grass tetany.

Experimental Procedure

During January 1966, 18 Hereford heifers averaging 185 kg. body weight were divided into three similar groups. All animals received grass hay *ad libitum*. Six animals served as controls and received grass hay only; six received 200 gm. of *trans*-aconitic acid plus 225 gm. potassium chloride (KCl) and six received 200 gm. of citric acid plus 225 gm. KCl in addition to grass hay for 6 days. The experimental acids and salts were suspended in water and administered by stomach tube within 5 min., daily. In subsequent studies the acids and salts were administered in a like manner.

In a second study, 12 similar animals were divided into three groups and given the following daily: grass hay only, grass hay plus 50 gm. citric acid or grass hay plus 50 gm. of *trans*-aconitic acid for 3 weeks (period 1). During a second 3-week period, the level of acids was increased to 100 gm. and half of the animals of each treatment also received 125 gm. KCl. For the third 3-week period the level of acids was increased to 150 gm. with KCl administered at the rate of 175 grams. For a fourth 3-week period the level of acids was increased to 200 gm. with half of the animals receiving 225 gm. of KCl in addition.

Since mature, lactating animals are more susceptible to grass tetany than heifers (Dozsa, 1959), a third study was inaugurated with animals of this description or approaching parturition at the Knoll Creek Field Laboratory of the University of Nevada in May, 1966. Animals were matched according to weight and assigned to one of the following three treatments: controls, no treatment; 500 gm. *trans*-aconitic acid plus 500 gm. KCl or 500 gm. citric acid plus 500 gm. KCl, but treated once at random.

In a pilot study initiated to distinguish between the effect of different treatment components, two animals were administered citric acid at the rate of 157 gm. per 100 kg. body weight; two additional animals received citric acid at the same rate with 157 gm. KCl per 100 kg. body weight and two received 194 gm. each of citric acid and KCl per 100 kg. body weight. Only one administration of citric acid and KCl was given.

A fourth study was designed to extend the previous observations and delineate the cause of this induced tetany. Thirty yearling cattle (average weight 237 kg.) were assigned at random to the following five treatments: KCl, citric acid, *trans*-aconitic acid, KCl plus citric acid and KCl plus *trans*-aconitic acid. All materials were administered once per animal at the rate of 157 gm. per 100 kg. body weight. These cattle were splenectomized, anaplasmosis carriers from a previous unrelated study. The basal diet prior to treatment was grass hay *ad libitum*.

In all studies except where indicated differently, KCl was administered with these acids in approximately equivalent amounts, since it was postulated that these acids would be found

[1] Supported in part by the Southwest Branch, Soil and Water Conservation Research Division, U.S.D.A. Approved for publication by the Nevada Agricultural Experiment Station as J.S. 97.

TABLE 1. THE EFFECT OF ORGANIC ACIDS AND POTASSIUM CHLORIDE ON YEARLING HEIFERS

Treatments	Controls	KCl plus *trans*-aconitic acid[a]	KCl plus citric acid[a]
No. of animals	6	6	6
Plasma Mg, mg./100 ml.			
Jan. 18, 1966 (initially)	1.87	1.85	1.73
Jan. 24, 1966	1.45	1.51	0.98[d]
Difference	0.42	0.34	0.75[d]
Plasma Ca, mg./100 ml.			
Jan. 18, 1966	10.9	10.8	11.4
Jan. 24, 1966	11.1	10.5	10.5
Difference	—.2	0.3	0.9
Deaths	0	2[b]	1[c]
Daily hay consumption, kg.	3.7[d]	1.6	1.6

[a] Two hundred grams of acid and 225 gm. KCl administered once daily *per os*.
[b] Died within 6 days.
[c] Died at 12 days.
[d] Significantly different from other treatments at $P<.01$.

primarily as the potassium salts in plant material but potassium *trans*-aconitate was not available. Plasma samples were taken initially and at various intervals throughout these studies. If tetany occurred, an attempt was made to treat the affected animal with a solution of calcium (Ca)-Mg. gluconate[2] intravenously after an additional blood sample was obtained. Plasma Mg was determined on an atomic absorption spectrophotometer (Beckman). Plasma Ca was determined by oxalate precipitation and subsequent titration with permanganate.

Results and Discussion

The data from the first experiment are shown in table 1. Citric acid plus KCl significantly decreased plasma Mg but had no significant effect on plasma Ca. Both organic acids with KCl decreased feed consumption ($P<.01$). Tetany was not observed in any of the animals. Autopsies were performed on the animals that died. Large areas of ecchymotic hemorrhage were observed on the ventral portion of the rumen, abomasum and part of the small intestine for the animals receiving *trans*-aconitic acid. In addition, marked autolysis of the kidney occurred. Except for the kidney lesions, the autopsy findings were similar for the animal receiving citric acid.

The organic acids had no measurable effect on plasma Mg nor was an interaction (KCl × organic acids) evident during the second study; hence the data are pooled and presented for KCl only (table 2). The addition of increasing amounts of KCl gradually decreased feed consumption and plasma Mg. These effects were more pronounced at higher levels of KCl and statistically significant. No death losses or visible symptoms occurred. Serial blood samples taken at 2- to 4-day-intervals indicated that the effect of KCl on plasma Mg occurred gradually and was not the effect of a single dosage. With the more prolonged administration of the KCl and organic acids, both acids behaved similarly, unlike the observation for the shorter term study. This suggests that some adaptation to the relatively large dosage of organic acids may occur. Potassium salts have been implicated in lowering plasma Mg and feed intake in other long-term studies (Rook and Storry, 1962; Kunkel, Barns and Camp, 1953). In the current study, treatments had no significant effect upon plasma Ca.

In the third study, 17 animals were treated as indicated in table 3. During this process, one treated animal died before being observed, while another was observed staggering. Muscular tremors occurred later, followed by the ani-

[2] Corvel Division, Eli Lilly and Co., Indianapolis, Ind.

TABLE 2. THE EFFECT OF POTASSIUM CHLORIDE ON FEED INTAKE AND PLASMA MAGNESIUM AND CALCIUM

Period	KCl daily	Plasma Mg.		Plasma Ca		Daily feed intake	
		KCl	No KCl	KCl	No KCl	KCl	No KCl
	gm.	mg./100 ml.		mg./100 ml.		kg./animal[c]	
1	0	1.63	1.55	11.6	11.4	4.1	4.4
2	125	1.43	1.62	11.6	11.6	3.8	4.9[b]
3	175	1.60	2.05[a]	10.5	10.8	3.6	4.6*
4	225	1.23	1.84*	9.2	9.4	3.1	4.4**

[a] Approaches significance ($F=5.85$ at P .05=5.99).
[b] Approaches significance ($F=5.51$ at P .05=5.99).
[c] Average weight 189 kilograms.
* Difference is statistically significant at $P<0.025$.
** Difference statistically significant at $P<0.01$.

TABLE 3. THE EFFECT OF ORGANIC ACIDS ON THE OCCURRENCE OF TETANY IN CATTLE[a] (KNOLL CREEK FIELD LABORATORY, UNIVERSITY OF NEVADA, 1966)

Item	Controls	KCl plus *trans*-aconitic acid[e]	KCl plus citric acid[e]
Body weight, kg.	390	355	369
No. treated	6	4	7
No. tetany[f]	0	2	4
No. died	0	1	2
Plasma magnesium		mg./100 ml.	
Initially	2.07	2.34	2.41
Prior to injection	....	2.60	2.32
24 hr. later	1.82	2.00	1.53
Difference at 24 hr.	0.25[b]	0.34[c]	0.88[d]

[a] Mature lactating cows or cows approaching parturition.
[b, c, d] Figures with different superscripts differ significantly ($P<.05$) within the same category.
[e] 500 gm. organic acids plus 500 gm. KCl daily *per os*.
[f] Tetany usually occurred within an hour of treatment.

mal falling one or more times. Later several additional animals showed a similar sequence of symptoms. When an animal could be approached while down, a solution of Ca-Mg gluconate was injected intravenously. Most animals recovered with one injection, although some animals had relapses and a second or third injection was necessary. Animals died if injections were initiated too late or not at all. The symptoms were similar to those observed with grass tetany in field cases and as described in the literature (Dozsa, 1959). The incidence of tetany between the two organic acids was similar.

Animal body weight varied widely within experimental treatments. Since a constant amount of organic acids and KCl was administered per animal, the dosage per unit of body weight was quite variable. This was examined as it related to the induced tetany. The animals that received the organic acid plus KCl and showed tetany received an average of 157 gm. per 100 kg. body weight, whereas the animals that received the same treatments but did not show tetany averaged 121 gm. per 100 kg. body weight. Data for the two acids were similar. In the earlier studies (tables 1 and 2), when no tetany was observed, the heifers received 108 gm. of these materials per 100 kg. body weight. Thus, it appears that the amount of these materials necessary to induce tetany is related to body size.

Blood samples were obtained before treatment, immediately before injecting Ca-Mg gluconate when tetany was observed and 24 hr. after the initial blood sample. When animals were in tetany, plasma Mg was similar to pre-treatment levels. Rook and Storry (1962) have indicated that plasma Mg tends to rise with the onset of convulsions; hence an analysis for Mg at this time may have limited value. Twenty-four hours after treatments were administered, the plasma Mg had decreased in all cattle but to a significantly greater extent in animals receiving organic acids and KCl. These data are similar to those obtained with the heifers in the first study. Both of these studies indicated that citric acid has a much greater effect than *trans*-aconitic acid on lowering plasma Mg when administered with KCl and measured in a short period post-administration. Plasma Ca was not affected by the experimental treatments. Plasma samples taken immediately after the animals showed tetany or 24 hr. post-treatment were slightly higher than pre-treatment levels. Samples taken after injection of Ca-Mg gluconate were much higher in both Ca and Mg as expected.

In the pilot study, the cattle that received citric acid alone showed no symptoms. When ctiric acid was administered with KCl (157 gm./100 kg.), tetany occurred but cattle responded to injections of Ca-Mg gluconate. When citric acid and KCl were administered at the higher level, tetany occurred but the animals failed to respond to Ca-Mg gluconate injections.

The results of the fourth study are shown in table 4. KCl or either acid administered individually had litttle effect on the incidence of tetany. However, tetany occurred in 66 to 100% of the cattle that received the combination of KCl and either acid. Again, the similarity of response of animals to citric or *trans*-aconitic acid suggests that their biological effect on the animal at these dosage levels is similar. These animals were not as responsive to intravenous injections of Ca-Mg gluconate as was noted in previous studies. No immediate explanation is available for this difference, but additional studies are under way to ascertain the causative factors.

If tetany was not observed in an animal, an additional blood sample was obtained approximately 2 to 3 hr. post-treatment. Treatment had no significant effect on plasma Mg when measured at this short interval. In general, the level of plasma magnesium was slightly higher when measured at tetany or 2 to 3 hr. post-treatment. Plasma Ca followed a similar trend as plasma Mg.

A combination of organic acids with KCl

TABLE 4. THE EFFECT OF ORGANIC ACIDS AND POTASSIUM CHLORIDE ON INDUCING TETANY IN CATTLE

Treatment[a]	KCl	Citric acid (CA)	*Trans*-aconitic acid (TAA)	KCl + CA	KCl + TAA
Cattle, no.	6	6	6	6	6
Tetany, no.	0	1	0	6	4
Deaths, no.	0	0	0	3	3
Treated, recovered, no.	..	1	..	1	1
Tetany, no treatment, no.	..	..	..	2	..
Plasma magnesium, mg./100 ml.					
Pre-treatment	1.94	2.01	1.92	1.90	2.09
Post-treatment[b]	2.08	1.98	2.06	2.12	2.5C
Plasma calcium, mg./100 ml.					
Pre-treatment	11.8	12.5	11.6	11.6	10.9
Post-treatment[b]	12.8	15.2	13.3	15.6	13.2

[a] All materials, where indicated, were administered once per *os* at the rate of 157 gm./kg. body weight.
[b] At tetany or 2 to 3 hr. post-treatment.

produces a tetany similar to grass tetany when administered at appropriate levels. Several theories have been suggested to explain this result but data supporting each is inadequate.

Summary

A tetany that resembles field cases of grass tetany has been experimentally induced in cattle by orally administering KCl plus either citric or *trans*-aconitic acid. These two acids or KCl administered separately do not produce tetany. The mechanism whereby this condition occurs is not delineated. Administration of KCl alone lowers plasma magnesium over extended periods, but the combination of KCl and citric acid lowers plasma magnesium in 24 hr. to 1 week.

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THE EFFECT OF A PROLONGED INTAKE OF PHOSPHORIC ACID AND CITRIC ACID IN RATS

BY

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Introduction

The use of orthophosphoric acid as an acidulant in refreshing beverages has become rather widespread. Therefore the action of this acid needs to be more thoroughly investigated. Although the capacity of the body to maintain its acid-base equilibrium, notwithstanding a considerable intake of acid or base, is an established fact, the question arises whether a prolonged intake of acid or base would be equally well handled.

The following mechanisms seem to be most important for the maintenance of the acid-base equilibrium with continuous acid ingestion: 1. the excretion of ammonia by the kidney. 2. the excretion of monobasic phosphate instead of dibasic phosphate by the kidney, the so-called "phosphate shift". 3. the excretion of acid calcium phosphate via kidney and intestine instead of intestinal excretion of neutral calcium phosphate (15, 27). Only when these mechanisms failed would an acidotic condition be expected, as well as a possible loss of fixed base from the body tissues.

The literature on this subject is rather inconclusive, as very little work has been done with phosphoric acid. *Tatsuzawa* (22) found a marked disturbance of the liver function in rabbits fed NaH_2PO_4. Dental erosion occurred in rats receiving phosphoric acid in the drinking water (16, 18). This erosive action, however, is not specific for phosphoric acid, as the same effect is produced by fruit juices (12, 17, 26).

It was therefore, decided to carry out experiments with rats over the whole life span and with successive generations in order to investigate the effect of phosphoric acid and citric acid on growth, reproduction, the blood picture, the histological appearance and chemical composition of different organs, and the effect on mineral and nitrogen metabolism. The effect of citric acid was studied for the purpose of comparison. Full details about techniques and results are given in a dissertation by one of us (3).

Experimental

Diets

As this experiment was conducted in the Netherlands, the basal diet (diet A) was designed to resemble the average Dutch diet as closely as possible, especially with regard to the acid-base balance. It is slightly acidogenic, as shown by the fact that the urine of the

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rats on this diet had an average pH of 5.87. This diet contained 0.60 % Ca and 0.82 % P.

Three experimental diets were used: basal diet plus 0.40 % phosphoric acid (diet B), basal diet plus 0.75 % phosphoric acid (diet C), and basal diet plus 1.20 % citric acid (diet D) (see table 1). The corresponding designations will be given to the groups of animals receiving these diets. A concentration of phosphoric acid of 0.5 % would provide, on the basis of body weight, the maximum acid intake in the human diet possible if the entire daily caloric requirements were obtained solely from the sugar present in the phosphoric acid-containing soft drink. Analysis of one of the most popular of these beverages showed that it contained about 0.055 % H_3PO_4 and 11 % sucrose.

Table 1. Composition of the diets

Basal diet (diet A):			
Casein	6.0 %	Brewer's yeast	5.0 %
Dried full milk	10.0 %	$Ca_3(PO_4)_2$	1.9 %
Whole wheat flour	34.5 %	$Na_2HPO_4.2H_2O$	0.8 %
Potato flour	33.0 %	KCl	0.9 %
Peanut oil	6.5 %	$MgCO_3$	0.2 %
Cod liver oil	0.5 %	Na-citrate	0.1 %
Linseed oil	0.5 %	Various salts[1])	0.1 %

Diet B = basal diet A plus 0.40 % phosphoric acid.
Diet C = basal diet A plus 0.75 % phosphoric acid.
Diet D = basal diet A plus 1.20 % citric acid.

Blood picture

Blood samples were obtained by amputating the tip of the tail (7, p. 351) after dilating the tail vein by rubbing the tail with toluene. The erythrocyte number was determined by measuring the extinction of the 20-fold diluted blood at 650 mμ (13).

Teeth

The method of *Keyes* (14) as modified by *Jansen* et al. (11) was used for the quantitative appraisal of the caries. The degree of dental erosion was also observed. Histological preparations were made of some of the molars by the method of *Mann* as modified by *Dobell* (20).

Urine

During the metabolism experiments the animals were kept in metabolism cages and the urine was collected separately from the feces over periods of seven days. The titratable acidity was determined according to *Henderson* and *Palmer* (8). For ammonia and urea the microdiffusion technique (5) was used. Total N was determined with the Kjeldahl technique, and inorganic phosphate by the method of *Sumner* (21). Calcium was determined according to *Wang* (24). Total base was determined by an ion exchange method (4). For citric acid the method of *Weil-Malherbe* and *Bone* (25) was used.

[1]) According to Jansen et al. (10).

Feces

The feces were dried overnight at 105° C, weighed and ground. Fecal total phosphorus was determined in a nitric acid-perchloric acid digest by means of the method of *Sumner* (21). Calcium was determined in the same digest with the method of *Wang* (24). Total N was determined with the *Kjeldahl* technique.

Blood

The animals were killed by decapitation and the collected blood was centrifuged immediately. The inorganic phosphorus was determined by the method of *Sumner* (21) and calcium by means of the procedure of *Wang* (24). For total base an ion exchange method (4) was used. The manometric method of *Van Slyke* (19, p. 267) was used for determining the total carbon dioxide content of the serum. Chloride was determined with an iodometric method (23). The method of *Bessey* et al. (2) was employed for the determination of alkaline phosphatase activity in serum.

Tissues

Total phosphorus was determined in a nitric acid-perchloric acid digest by means of the method of *Sumner* (21). In the same digest Na and K were determined flame photometrically, using blanks and standards containing approximately the same amounts of nitric and perchloric acid as were present in the digests. The alkaline phosphatase activity of the kidney was determined by the method of *Bessey* et al. (2) in a 2.5 % homogenate.

The tibiae were ashed at 600° C after extraction of the lipids with ether ethanol. The ash was dissolved in dilute hydrochloric acid and the phosphorus determined with the method of *Sumner* (21) and the calcium with *Wang*'s (24) method.

Statistical analysis

The statistical methods used in the evaluation of the results have been described in detail (3). The bilateral tail probability (p), i.e. the probability that the difference found be accidental, was calculated in each instance. The difference was considered significant, whenever p was smaller than 0.05. Values of p in text and tables have been printed in italics.

Results

Biological experiments

The average weights for each experimental group at 0, 10 and 20 weeks after weaning were compared. Diets containing either 0.40 % or 0.75 % phosphoric acid or 1.20 % citric acid had no harmful effect on the growth of three successive generations of rats fed these diets.

The reproduction of the rats in dietary groups A, B and D was investigated when they were 32 weeks old and had received the diets for 29 weeks. Eleven weeks later the same animals were mated again. No detrimental effect of the acid diets on the reproduction existed, either

after the first, or after the second mating, as judged by the weight of the mothers, the number of living young and stillborn per litter, the average birth weight of the living young, and the number of young left at weaning.

The blood picture was determined in 20 animals of the first generation and in 20 animals of the second generation. In each case 10 animals of group A and an equal number of group B, including both male and female animals were investigated. The age was 32 weeks and the diets had been given for 29 weeks. Erythrocytes, leucocytes, lymphocytes, neutrophiles, eosinophiles, basophiles, young leucocytes and monocytes were counted. No significant differences were found for any of the elements. No gross anatomical changes were found in any of the animals except for more or less marked pulmonic lesions due to pneumonia, which were of the type described by *Griffith* and *Farris* (7, p. 460-2) and occurred with about the same frequency in control and experimental groups.

Histoligical examination of the liver, spleen, adrenals, testicles, skeletal muscle and femur revealed no pathological changes or any peculiarity. As in some cases abnormalities of the kidney were found, the investigation of this organ was extended to additional animals. The abnormalities were found to occur with practically the same frequency in the controls as in the experimental animals and therefore could not be attributed to the acid diets. Similar lesions of the kidneys with about the same frequency were discribed by *Griffith* and *Farris* (7, p. 469) and labelled as spontaneous diseases. A possible explanation of the changes found may be degeneration due to chronic infections, e.g. pneumonia.

In a study of the teeth of a number of rats of the dietary groups A, B, C and D (diets during periods from 3 to 16 months), no extensive lesions were found only some fissural caries. The caries percentage, i.e. the relative amount of dental tissue lost due to a carious process, was exceptionally low (about 0.1 %) and about the same for all dietary groups.

In all groups, dental attrition of the molars was found to occur with increasing age. The highest degree of attrition was found in the animals receiving phosphoric acid and the lowest in the animals of group A. No caries was observed in the abraded cuspal parts. Apparently there was no connection between caries and attrition, the former always being fissural and independent of the acidity of the diet. A remarkable feature of the molars of the animals receiving phosphoric acid was the white colour, which was presumably due partly to the proper colour of the molars and partly to the reduced plaque.

On histological examination of the teeth the tubules of the dentine of the animals receiving the acid diets were found to be filled with a calcium-containing substance, differing from normal dentine by being somewhat translucent. This is probably an indication of a reaction of the dentine to the acid diets. This process had not progressed to a

further degree in the animals receiving diets B and D for 10 months compared to the situation after 3 months (6 specimens). Neither after 3 nor after 10 months was there any indication of a reaction of the pulp; only normal odontoblasts were found. No secondary dentine formation had occurred. In the histological examination no traces of caries were apparent, which is in accordance with the very low caries percentage. From these observations it was concluded that the increased attrition was not to be regarded as a harmful action of the acid diets.

Metabolism experiments

In experiment 1, 24 female rats of the control group (age 8 months) were kept on control diet A for the first 6 weeks of the experiment. During the next 8 weeks 8 animals were continued on this diet, while 8 others received the diet B containing 0.40 % phosphoric acid, and the remaining rats received diet D containing 1.20 % citric acid. The experiment was completed with another control period of 6 weeks during which all animals received diet A. As this experiment could only yield information over a relatively short period of acid administration, another metabolism experiment (experiment 2), in which 8 animals of dietary group A were compared over five weeks with 8 animals of dietary group B, was carried out. All animals were 8 months old at the start and had received the diet of their group since weaning. The Ca-, P-, and N-balances, determined in these experiments, are the differences between the intake of these elements on the one hand and their excretion in urine and feces on the other hand, expressed in mg/day.

Table 2. Phosphorus and calcium balance and excretion

Diet group	Phosphorus				Calcium			
	Balance mg/d	Urine mg/d	Feces mg/d	Ratio urine/fecal P	Balance mg/d	Urine mg/d	Feces mg/d	Ca/P in feces
A	22.1	35.6	68	0.56	18.7	1.08	73	1.13
B	27.2	43.2	68	0.65	22.2	0.97	67	1.00
p*)	*0.044*	*0.130*	*0.646*	*0.105*	*0.177*	*0.195*	*0.146*	*0.083*

*) In this and subsequent tables the values for p, printed in italics, are the bilateral tail probabilities resulting from the statistical treatment.

Table 3. Nitrogen balance and excretion

Diet group	Balance mg/d N	NH_3-N in urine mg/d	Urea-N in urine mg/d
A	61	7.5	114
B	60	10.6	129
p	*0.959*	*0.0087*	*0.130*

In table 2 the phosphorus balance and excretion as well as the calcium balance and excretion for experiment 2 are given. In both experiments the phosphorus balance was increased by the phosphoric acid diet. Also increased was the urinary phosphorus output, though significantly only in experiment 1. The fecal P excretion remained unchanged in both experiments and consequently the ratio urinary/fecal P excretion increased, significantly only in experiment 1. The citric acid did not exert any influence on the phosphorus balance or excretion. Neither of the two acids caused a significant change in the calcium balance and excretion in either experiment. Notably the urinary calcium output did not increase. However, the fecal Ca/P ratio had the tendency to decrease for group B in both experiments. Combination of the two probabilities, according to the method of *Fisher* (6, p. 99), showed the effect to be significant ($p = 0.033$). This would mean that in the phosphoric acid group acid calcium phosphate replaced part of the $Ca_3(PO_4)_2$ excreted in the feces.

The results obtained for the nitrogen balance and excretion in experiment 2 are given in table 3. In experiment 1 only the urinary ammonia and urea excretion were determined. The phosphoric acid diet did not affect the nitrogen balance, although a very significant increase in the urinary ammonia excretion occurred ($p = 0.0087$). Simultaneously, however, there is an indication of a decrease of the urea excretion, sufficient to compensate for the extra ammonia excretion. In experiment 1 the phosphoric acid group showed a similar increase in the ammonia excretion, while the sum of urinary ammonia and urea nitrogen did not change. The citric acid group showed no effects on the ammonia and urea excretion. These results can be explained as follows: in our diets the protein supply was abundant and therefore enough glutamine was available for the formation of ammonia. Thus, the only effect was a decreased urea excretion, while the nitrogen balance remained positive.

Table 4 shows the fixed base excretion in experiment 1. The ion exchange method used determined ammonia in addition to Na, K, Ca and Mg; the total base content minus the ammonia content therefore gave the fixed base concentration of the urine. It is clear that neither of the acid diets had any effect on the urinary fixed base excretion, so the acid diets did not cause a loss of fixed base from the body tissues.

Table 4. Fixed base excretion

Diet group	CP 1*) meq/d	EP meq/d	CP 2 meq/d
A	3.67	3.63	3.67
B	3.52	3.63	3.63
D	3.36	3.52	3.70

*) In this and subsequent tables CP 1 and CP 2 are the control periods, EP the experimental period.

Diet group	CP 1 vs. EP	EP vs. CP 2
p A vs. B	*0.959*	*1.000*
p A vs. D	*0.161*	*0.955*

Table 5. Volume, titratable acidity and pH of urine

Diet group	Volume ml/d	Titratable acidity meq/d	pH
A	6.8	0.40	5.89
B	5.4	0.53	5.66
p	*0.028*	*0.013*	*0.0019*

In table 5 the daily volume, titratable acidity and pH of the urine in experiment 2 are presented. The titratable acidity was raised and the pH was lowered by the phosphoric acid in both experiments. The citric acid did not change the titratable acidity and the pH significantly. The daily volume of urine was unaffected in experiment 1 by either of the acids, while in experiment 2 it was lowered by the phosphoric acid, so there was no diuresis.

Table 6. Citric acid in urine

Diet group	CP 1 mg/d	%	EP mg/d	%	CP 2 mg/d	%
A	6.1	76	8.4	99	11.6	126
D	5.7	67	11.0	5.3	12.2	137

Diet groups	CP 1 vs. EP	EP vs. CP 2
p A vs. D	*0.758*	*0.805*

The urinary citric acid excretion for groups A and D in experiment 1 is given in table 6, both in mg/d and in per cent of the total intake (the basal diet contained 0.051 % citric acid as Na-citrate). The statistical comparison was carried out for the excretion in mg/d. No increased citric acid excretion was found. Apparently the animals could oxidize almost all of the extra ingested citric acid, assuming that no appreciable amounts were excreted in the feces or deposited in the body.

Tissue analyses

The experiments presented in table 7 consisted of two series: in series 1 twelve 15-month old female rats from each of the dietary

Table 7. Tissue analyses

Diet group	Blood serum					Kidney		
	Inorganic P	Ca	Total base	Cl	Alkal. phosphat.	Alkal. phosphat.	Water content	Ash content
	mg%	mg%	meq/ml	mg%	mM units/ml	mM units/mg	%	%
Series 1								
A	7.0	10.4	0.123	336	6.0	1.09	76.8	1.46
B	6.3	10.7	0.120	333	5.3	0.82	77.1	1.54
D	5.9	11.7	0.121	340	5.1	0.78	77.9	1.34
Series 2								
A	8.1	9.4	0.121	331	18.7	2.75	76.6	1.16
B	7.7	8.8	0.116	333	17.9	2.31	76.6	1.12
D	8.1	9.2	0.117	337	15.8	2.46	77.0	1.14

Diet group	Liver			Muscle			Tibia		
	Total P	K	Na	Total P	K	Na	Ash content	Ca in ash	P in ash
	mg/g	µeq/g	µeq/g	mg/g	µeq/g	µeq/g	% dry wt.	% ash	% ash
Series 1									
A	3.53	78	40	2.63	94	23	60.8	34.4	19.3
B	3.46	75	40	2.57	95	23	58.0	33.4	19.2
D	3.38	73	37	2.43	91	23	58.2	36.9	19.2
Series 2									
A	3.69	81	29	2.63	98	23	64.3	34.2	19.2
B	3.60	80	33	2.63	96	25	62.7	35.6	19.0
D	3.66	81	31	2.36	85	28	63.3	34.7	19.3

groups A, B and D were used. In series 2 6-month old male rats of the second generation were used, 14 rats from group A, 15 from group B and 7 from group D.

No differences in either of the experimental groups of series 1 and 2 were found in inorganic phosphorus, calcium, total base, chloride and alkaline phosphatase activity in blood serum, in the fresh weight, ash content and alkaline phosphatase activity of the kidney, in fresh weight, total P and K of the liver, in K content of the muscle and finally in the ash content and P content of the tibia. A slight indication of a decreased water content of the kidney was found in group B of series 1 (the average 77.1 is high because of one very high value — 81.9 — in this group). Since no parallel increase in the ash content was found and also as no effect in series 2 existed, not too much importance can be ascribed to this finding. In any case there was no sign of renal edema or renal enlargement.

The Na content of the liver was decreased in both series by the citric acid. No change was found in the phosphoric acid group. The total P content in muscle was also decreased by the citric acid in both series, while no change was observed in the phosphoric acid group. The decrease in the total P content of the muscle may be due to a partial exchange of phosphate ions for citrate ions, or to a mobilization of phosphate for the oxidation of the citric acid ingested. An increase of the Na content of the muscle was found in both series for both acids, the increase being significantly larger for citric acid than for phosphoric acid. The absence of a decrease in the Na or K content of the soft tissues is in accordance with the absence of an increased fixed base excretion found in the metabolism experiments. The only difference found in the analysis of the tibia is an increased calcium content of the ash for the phosphoric acid group in series 2. There is no indication of a decalcification of the skeleton due to the acid diets.

In a separate experiment the total carbon dioxide content of the blood serum of 10 male rats of group A (age 12 months, first generation) and 10 male rats of group C (age 13 months, second generation) was determined with the following results:

group A 70.7 vol. % (standard error*) 2.2 vol. %)
group C 71.4 vol. % (standard error 2.0 vol. %)

Apparently even the extreme diet C (0.75 % phosphoric acid) did not cause acidosis in the second generation after one year.

Experiments with radioactive phosphorus

Two experiments with radioactive phosphorus were carried out to supplement the evidence obtained from the metabolism experiments and tissue analyses. In the first experiment the endogenous fecal phosphorus excretion was determined by means of the procedure of *Hevesy* et al. (9). Seven days after intraperitoneal injection of P^{32}-

*) Defined by the formula: S.E. $= \sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}$, where n represents the number of measurements (x), from which the average $\bar{x}$ has been calculated.

phosphate the urine and feces were collected in two periods of seven days. For each collection period the specific activities of urine and feces were determined, their ratio giving the percentage of endogenous phosphorus in the total fecal phosphorus excretion. Ten 7-month old female rats from each of the dietary groups A, B and D were used. The only significant change was an increased ratio of urinary to fecal phosphorus for the phosphoric acid group (table 8), a fact also found in the metabolism experiments. There was a tendency for a parallel rise in the ratio of the activities excreted daily in urine and feces, but no change in the endogenous fecal phosphorus excretion was found.

Table 8. Endogenous fecal phosphorus excretion

Diet group	% endog. fecal P		urin./fecal activ.		urin./fecal P		total activ./day	
	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week
							μC/d	μC/d
A	20.1	22.1	2.09	1.85	0.40	0.38	0.30	0.15
B	25.9	20.8	2.65	2.73	0.50	0.50	0.31	0.15
D	32.6	19.2	2.34	1.88	0.48	0.35	0.37	0.16
p A vs. B	*0.549*		*0.296*		*0.025*		*0.796*	
p A vs. D	*0.971*		*0.825*		*0.631*		*0.123*	

In the other experiment the phosphorus retention of the tibia was determined in eleven 13-month old male rats of each of the groups A, B, and D. One hind leg was amputated at the beginning of the experiment, 24 hours after intraperitoneal injection of P^{32}phosphate, to serve as an internal reference standard. After six weeks the animals were killed, and the other tibia obtained. The specific activities in both tibiae were determined, their ratio giving the retention of phosphorus by the bone (1). The average phosphorus retentions of the tibiae over a six-week period were: group A 63 %, group B 63 %, group D 69 %. The differences between the groups are not significant ($p = 0.187$ and 0.346 respectively).

Discussion

The outstanding results of these experiments are the absence of an acidotic condition in the rats, the absence of a loss of calcium and the other fixed bases, and the absence of a loss of nitrogen from the body. The absence of changes in the mineral composition of the blood serum is in good agreement with the findings for the mineral metabolism. These results were obtained with both acids. The complete similarity between the results of the two metabolism experiments proves that the metabolic effects of the phosphoric acid after a prolonged intake were the same as after a relatively short one. The phosphoric acid was neutralized partly through the excretion of ammonia by the kidney,

partly through the phosphate shift resulting in an increased titratable acidity and a decreased pH of the urine, and partly through the excretion of a more acid calcium phosphate in the feces.

The increased retention of phosphorus probably resulted from an increased absorption due to the acid regime. The increased absorption with the resulting increase in phosphorus retention as well as the increased urinary phosphorus excretion were the only observable changes in the metabolism of this element. There was no difference in the turnover of the skeletal phosphorus, nor any change in the alkaline phosphatase activity of the blood serum and the kidney, nor any difference in phosphorus content of the body tissues. There was no indication whether the retained phosphorus was deposited in the skeleton or in organic compounds in the blood or the soft tissues.

The citric acid proved to exert even less effect on the mineral metabolism: the organism was able to oxidize all of the extra ingested citric acid. Some slight effects on the tissue composition were found: a decreased Na content of the liver, a decreased total phosphorus content and an increased Na content of the muscle. The increased Na content of the muscle also occurred in the phosphoric acid group.

Summary

Albino rats were maintained on diets to which either phosphoric acid or citric acid were added in quantities that represented, on the basis of body weight, the maximum daily intake of acids that would be possible if the entire daily caloric requirements were obtained solely from the sugar contained in soft drinks.

No harmful effects were produced by the acid diets in two successive generations of animals maintained on these diets for a considerable part of the life span, as shown by an extensive study of growth, reproduction, the blood picture, the gross and microscopical appearance of the organs, the mineral and nitrogen metabolism and the tissue composition.

The normal physiological processes for the removal of ingested acids were found to be sufficient even after a continuous intake in two successive generations.

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A Rachitogenic and Growth-Promoting Effect of Citrate [1]

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Introduction

Although the literature reveals certain limitations to the effective use of citrates as antirachitic agents for infants (1–6), it is well known that, for rats, citrates are definitely antirachitic when incorporated in adequate amounts in high-Ca cereal diets (7–9). Their action with cereal rations has been attributed to the formation of Ca complexes which prevent Ca from interfering with the hydrolysis of phytic acid (8, 10). However, with noncereal rations which have inorganic P as the source of P, citrates have been reported to be ineffective (11). Lately, however, an antirachitic effect has been obtained with these as well (12). In view of the ability of citrates to complex Ca, it remains to be seen whether they can be harmful instead of beneficial under dietary conditions other than those which have been studied.

In our presently reported experiments, citrates were incorporated in a strongly rachitogenic, low-P, adequate-Ca diet from which Ca is readily absorbed, but from the feeding of which a negative Ca balance is induced even when vitamin D is present (13).

Experimental

The diet fed was basically the noncereal low-Ca low-P (0.02%) diet of Bellin and Steenbock (14) modified by the addition of $CaCO_3$ to a Ca content of 0.50%. This diet was fed as such, and after the incorporation of 0.02 mole Na citrate and 0.02 mole citric acid in each 100 g. portion at the expense of glucose.[2] Both diets were fed, with and without vitamin D. Vitamin D was given to some of the rats

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[2] Cerelose.

once every 3 days in the form of a solution of 75 I.U. of crystalline vitamin D_2 in 0.2 ml. of cottonseed oil. Food intake was restricted to 20.0 g. per rat per 3 days so that direct comparisons of the results from individual rats could be made. As this restriction was expected to limit differences in the degree of response, the identical rations were also fed *ad libitum* to four additional groups of six rats each for comparative purposes.

The rats (Sprague-Dawley) weighed from 76 to 80 g. They were housed in individual wire metabolism cages. Urine and feces were collected at 3-day intervals for 9 days following a preparative feeding period of 3 days. To avoid contamination of the urine with ration and feces, the rats were transferred to separate feeding cages three times daily. The floors of these cages were provided with double thicknesses of 12 in. qualitative filter paper to prevent the loss of urine and feces excreted and ration spilled during the feeding period. At the end of the experiment, bladders and kidneys were inspected for calculi which, when found, were analyzed for Ca. These Ca values (0.4–7.9 mg.) were added to the urinary Ca values of the last 3-day period of collection. Lithiasis was incident only in the citrate groups in six out of eight rats when vitamin D was given, and three out of eight rats when it was not given.

Urine and feces were wet-ashed by nitric-perchloric acid digestion. Ca analyses were made according to the oxalate method of Wang (15), and P analyses by the method of Fiske and SubbaRow (16).

Results

It is seen (Table I) that, with an almost constant intake of Ca, citrate greatly increased the fecal excretion of Ca. When the absorption of Ca was at a high level, as induced by vitamin D, the comparative effect of citrate was less than it was under the more adverse conditions when vitamin D was not given. Under these latter circumstances citrate prevented completely the absorption of Ca in the early part of the experiment.

The Ca balances (Table II) were negative when vitamin D was given, as was to be expected from the work of Day and McCollum (13) and, as shown here, they were even more negative in its absence. In the latter case, citrate had no over-all effect beyond shifting part of the excretion from the urine to the feces. But when vitamin D was given, citrate counteracted the Ca-conserving effect of the vitamin, and the loss of Ca was increased though not to the level which prevailed in its absence. It is clear that the increase in the loss of Ca was caused largely by an increase in fecal excretion.

The P balances (Table II) can be evaluated only in relation to the data presented in Table III. It will be noted that citrate had no effect unless vitamin D was given. Under these latter circumstances the loss of P was substantially increased.

TABLE II

Effect of Citric Acid and Vitamin D on Ca and P Balances

The values given in the table are average values from six to seven rats in each group.

Period	Urinary Ca		Fecal Ca		Ca Balance		P Balance	
	With vitamin D	Without vitamin D	With vitamin D	Without vitamin D	With vitamin D	Without vitamin D	With vitamin D	Without vitamin D
days	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Without citric acid								
4th–6th	55.6	62.0	46.9	72.8	−3.8	−28.6	−3.1	−6.9
7th–9th	55.5	55.7	41.4	77.8	+2.0	−28.4	−1.8	−5.4
10th–12th	64.9	56.5	49.1	88.5	−8.6	−38.3	−2.0	−5.8
Total: 9 days	176.0 (140.0–215.5)	174.2 (161.8–187.5)	137.4 (89.8–181.0)	239.1 (223.5–255.5)	−10.4 (+13.8)–(−26.3)	−95.3 (−86.6)–(−101.0)	−6.9 (−5.1)–(−9.4)	−18.1 (−13.8)–(−20.4)
With citric acid								
4th–6th	53.0	37.7	76.1	107.0	−25.8	−38.2	−4.1	−5.2
7th–9th	61.7	32.8	61.0	111.1	−17.2	−36.5	−3.0	−4.9
10th–12th	51.8	29.8	57.2	91.6	−3.5	−14.2	−2.9	−3.1
Total: 9 days	166.5 (131.2–213.4)	100.3 (61.3–123.5)	194.3 (149.7–217.8)	309.7 (292.3–345.7)	−46.5 (−26.9)–(−68.1)	−88.9 (−31.6)–(−124.9)	−10.0 (−4.2)–(−14.3)	−13.2 (−10.5)–(−18.8)

TABLE I

Effect of Citric Acid and Vitamin D on Ca Absorption

The ration fed contained 0.5% Ca, as calcium carbonate, and 0.015–0.017% P. Citric acid was added as 0.02 mole Na citrate plus 0.02 mole citric acid per 100 g. of ration. The values given in the table are averages from seven rats with the exception of those for the group which received neither citric acid nor vitamin D. In this group the values from six rats were averaged.

Period	Ca intake		Net Ca absorbed	
	With vitamin D	Without vitamin D	With vitamin D	Without vitamin D
days	*mg.*	*mg.*	*mg.*	*mg.*
Without citric acid				
4th–6th	98.7	105.8	51.7	33.5
7th–9th	98.9	105.2	57.5	27.3
10th–12th	105.4	106.8	56.3	18.2
Total: 9 days	303.0 (289.9–320.4)	317.8 (302.3–320.4)	165.5 (131.0–200.1)	79.0 (64.9–90.0)
With citric acid				
4th–6th	103.2	106.5	27.1	−0.5
7th–9th	105.6	107.5	44.5	−3.6
10th–12th	105.3	107.2	48.1	15.6
Total: 9 days	314.1 (299.9–322.5)	321.2 (316.6–322.5)	119.7 (103.4–171.8)	11.5 (−23.2)–(+29.7)

The importance of the data in Table III in reference to the P balances lies in the comparative gain in the weights of the animals. Citrate had no effect on the gain in body weight in the absence of vitamin D, but when vitamin D was given, it reduced the well-known growth-depressing effect of vitamin D in rats fed adequate-Ca low-P diets (17, 18). Apparently the extensive withdrawal of Ca from the skeleton liberated large amounts of skeletal P needed for growth. It will be noted that the losses of P range in value in the same order as those of Ca, although because of the use of part of the P for growth they differ from the ratio in which Ca and P occur in the skeleton.[3]

[3] This ratio is approximately 2.27 as calculated from data on the femur published by A. E. Sobel, Morris Rochenmacher, and Benjamin Kramer, *J. Biol. Chem.* **152**, 255 (1944).

TABLE III

Effect of Citric Acid and Vitamin D on Growth, Bone Ash and Serum Ca and P

	Vitamin D	Body weight		Femur ash	Femur ash			Metaphyseal widths	Serum Ca	Serum inorg. P
		Initial	Gain		Initial[b]	Final	Loss			
Equalized intake[a]										
		g.	*g.*	*%*	*mg.*	*mg.*	*mg.*	*mm.*	*mg. %*	*mg. %*
Without citric acid	0	80	16	45.4	—	52.6	--	--	12.6 (2)	2.6 (2)
	+	79	6	51.2	—	65.3	--	—	15.8 (2)	3.8 (2)
With citric acid	0	76	18	45.7	—	52.9	—	—	12.0 (5)	4.4 (2)
	+	77	11	48.3	—	58.3	—	—	14.6 (4)	4.6 (2)
***Ad libitum* intake[c]**										
Without citric acid	0	77	40	29.4	58.5	42.1	16.4	1.6	11.9 (5)	2.4 (3)
	+	79	11	43.7	60.0	57.3	2.7	0.2	15.2 (3)	4.1 (2)
With citric acid	0	77	38	28.3	58.5	38.6	19.9	1.8	11.9 (5)	2.9 (2)
	+	77	33	35.8	58.5	48.3	10.2	0.4	15.9 (6)	3.7 (4)

[a] Average values obtained on the 12th day from six to seven rats in each group except when designated by lesser numbers enclosed in parentheses.

[b] Average value from five rats for each group as received from the breeders.

[c] Average values obtained on the 21st day from six rats in each group.

The evidence for a rachitogenic effect of citrate as indicated by the increase in the negative balance of Ca and P is supported by the reduction in bone ash as calculated on either a dry weight or percentage basis, and by the increase in the average width of the metaphyses. Serum Ca and P were not affected, probably because of the short duration of our tests.

Summary

In studies with rats it was found that the absorption of Ca from a vitamin D-free diet which contained adequate amounts of Ca (0.5%) and a low supply of P (0.015%) was greatly reduced by the incorporation of Na citrate and citric acid. When vitamin D was added, in addition to

the citrate, the absorption of Ca was increased but the level attained was still lower than when citrate was omitted. It is concluded from observed negative Ca and P balances, decreases in bone ash, and increases in metaphyseal widths that citrate had a rachitogenic effect.

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The Effects of Citric and Phosphoric Acids on the Teeth

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Dental caries was studied in albino rats which received various amounts of citric or phosphoric acid added to a dry, non-cariogenic food. The composition of this diet was, in percentages, as follows: whole-wheat flour, 34.5; potato flour, 33.0; dried whole milk, 10.0; casein, 6.0; peanut oil, 6.5; brewer's yeast, 5.0; salt mixture (including trace elements), 4.0; cod-liver oil, 0.5; linseed oil, 0.5. The diet was slightly acidogenic; the pH of urine was 5.87 ± 0.03.

Three groups of 68, 19, and 6 animals, respectively, were used. The animals of Group I ranged in age from 6 weeks to 7 months; those in Group II were 3 and 10 months old. The experimental diet was initiated in Groups I and II at weaning. The experimental animals in both groups received either 0.5, 1.0, or 1.5 gm. of phosphoric acid per kilogram of food or 1.5, 4.5, or 12.0 gm. of citric acid per kilogram of food. This 1:3 weight ratio between the acids (1:1.5 molar ratio) was based on the pH and the taste of the aqueous solutions. The animals in Group III were 1 year old at the time of caries evaluation and 2 months old at the time the diet was initiated. The experimental animals in this group received either 75 mg. of phosphoric acid or 312 mg. of citric acid per 10 gm. of the basal ration. Control animals were included in all three groups. Non-decalcified, fuchsin-stained sections of the jaw quadrants were subsequently studied for caries under a microscope (*Intern. Z. Vitaminforsch.*, **26**:235, 1955).

None of the experimental animals showed severe lesions with defects of the enamel continuity in the fissures or approximal surfaces. One of the control animals had a severe lesion in the second upper molar on the distobuccal site, and it appeared to be a smooth-surface lesion. All the fissure abnormalities were confined to a slight staining with fuchsin; whether this condition was pathological or only a sign of not yet fully matured dental tissue was not determined. The approximal lesions were chalky white, with loss of enamel translucency. None of the acid-supplemented groups developed more caries, on the average, than did the controls. However, the molars in rats on the acid-supplemented diets, especially those in animals receiving citric acid in the highest dosages, showed more wear and attrition than did the molars of the control animals. Shallow or nearly absent fissures resulted in the older age groups. It was concluded that the enamel-staining properties were significantly fewer in the two older groups than in the younger groups. No distinct differences were noted in the fissure dentino-enamel scores, but scores of approximal enamel lesions were significantly higher in the 7-month-old group than in the younger animals.

The results in Group II were similar to those for Group I. The enamel scores in the 10-month-old rats were significantly lower than in the 3-month-old group. The differences between the fissure dentino-enamel junction scores for both age groups were not statistically significant, nor were those of the approximal surface attack.

After 1 year, no severe lesions had developed in Group III, in spite of the fact that these animals were started on the non-cariogenic ration when they were 2 months old, the age prior to which they had been fed a cariogenic stock diet known to cause many initial carious lesions in older animals within a 2-month period.

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International Association for Dental Research

Organized: New York City, December 10, 1920. Of those at the meeting of organization, the following remain active members: Adolph Berger, Theodor Blum, G. W. Clapp, A. H. Merritt, B. B. Palmer, L. M. Waugh.

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Citrate Metabolism During Surgery

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All postoperative patients are temporarily diabetic, at least with respect to the assimilation of exogenous glucose.[1] Generally, the severity of the change in glucose tolerance is proportional to the magnitude and extent of the operative procedure.[2] Unless the postoperative period is complicated by an infection or by conditions that preclude optimal alimentation, a normal tolerance for glucose is reestablished usually within a week. In marked contrast, there is no impairment in the rate of assimilation of fructose either during or after surgery in comparison with the preoperative rate.[1]

One interpretation of these findings is that surgical stress has some effect on factors responsible for the cellular transport of glucose, but it has no effect on the permeability of the cell wall to fructose. Another interpretation is that stimulation of the pituitary-adrenal axis results in a humoral inhibition of glucokinase with a consequent delay in the entrance of glucose into the metabolic pool. The separate hexokinase of fructose may not be influenced by the postoperative altered humoral environment and thereby may provide for a normal disappearance of the administered fructose.

More recently, surgical stress has been found to alter the metabolism of both pyruvate and lactate.[3] In view of the postoperative decreased rate of assimilation for glucose, pyruvate, and lactate, but not for fructose, the suggestion was made that enzymatic processes are not uniformly disturbed by surgery.[4] Rather, surgical stress alters specific metabolic pathways. Clearly, additional study is indicated to help elucidate the mechanisms by which surgery alters intermediary metabolism.

Previous studies have concerned substances whose initial metabolism involves anaerobic reactions in the Embden-Myerhof scheme of glycolysis.[1,3] Since most of the energy produced by the metabolism of glucose is derived ultimately from reactions in the aerobic Krebs cycle, it seemed reasonable to study the integrity of these pathways. Accordingly, the present investigation of oxidative metabolism was made in surgical patients by a study of citrate tolerance.

Methods

A series of 3 citrate-tolerance tests were performed on each of 5 patients who underwent elective operations with general anesthesia. For several days prior to the study, care was taken to provide an adequate diet with supplementary vitamin-B complex. Alimentation during and following surgery was supplemented by intravenous infusions to insure an intake isocaloric with the preoperative diet and thereby to prevent a "starvation" type of diabetes postoperatively.[5] The tests were performed after the subject had rested for 12-18 hours.

The citrate tolerance tests were conducted by the intravenous administration of a 2½% solution of sodium citrate with a constant infusion pump for a period of 1 hour. The rate of administration was calculated on the basis of 0.065 gm. of sodium citrate per kilogram of body weight per hour. In a series of preliminary studies this amount of citrate was found not to cause an alteration in electrocardiographic pattern or in the blood level of total calcium. The concentration of citrate in venous blood rose from 2 to 7 mg. per 100 ml., a rise insufficient to produce symptoms of clinical tetany. It was calculated to provide a quantity of citrate for an average-sized adult, generally equivalent to that found in one liter of bank blood.

Samples of venous blood were obtained through an indwelling Cournand needle prior to the start of

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TABLE 1.—*Comparison of Fasting Concentration of Carbohydrate Intermediaries Before and After Surgery, Mg. per 100 Ml.**

	Mean	S.E.M.	N	p
Glucose				
Preop.	85.8	±2.5	12	$0.005 > p > 0.001$
Postop.	109	±7.2	12	
Lactic Acid				
Preop.	8.4	±0.76	12	$0.01 > p > 0.005$
Postop.	12.1	±0.95	12	
Pyruvic Acid				
Preop.	0.89	±0.07	12	$0.05 > p > 0.025$
Postop.	1.20	±0.11	12	
Citric acid				
Preop.	2.16	±0.79	12	$p > 0.8$
Postop.	1.89	±0.95	12	

* Abbreviations: S.E.M. indicates standard error of the mean; N, number of subjects.
Consider values with $p < 0.05$ significantly different.

the citrate infusion and every 30 minutes thereafter for a total of 3 hours. The blood samples were analyzed for glucose and for pyruvic, lactic, and citric acids. Urine was collected for the 3-hour period of the test, but the quantity of citrate excreted was small and did not vary significantly between the 3 tests. Citrate was determined as citric acid by the pentabromoacetone method of Taussky as modified by Natelson.[6] The chemical methods for the determination of glucose and of pyruvic and lactic acids have been described previously.[4]

Twelve patients were studied for the effect of surgery on the fasting blood concentration of glucose and carbohydrate intermediaries. The control specimens were obtained 1 or 2 days prior to surgery, and the postoperative specimens were obtained on the first day after surgery. All operations were performed under general anesthesia exclusive of ether.

In order to determine the reproducibility of the citrate tests, 2 subjects not operated on had citrate-tolerance tests performed on 3 different days. The results of each of these series of 3 control tests are recorded in Table 2. When the study of the effect of surgery on citrate metabolism was undertaken, the initial, or control-tolerance test, was completed 1 or 2 days prior to surgery. The second test was started one-half hour after the commencement of the operative procedure. The third and final tolerance test was performed early in the morning of the first postoperative day.

Four of the patients were anesthetized with thiopental sodium (Pentothal), nitrous oxide, and cyclopropane; 1 patient had a spinal anesthetic. Previous studies showed that the quantity of thiopental sodium used in these tests does not cause an alteration in carbohydrate metabolism.[4] Also more recently we have not found a disturbance in carbohydrate metabolism during cyclopropane anesthesia. The 5 operations included 2 subtotal thyroidectomies for nontoxic multinodular goiter, an abdominal laparotomy for an appendectomy and bilateral oophorectomy, an extensive pedicle flap for repair of a leg ulcer, and a bilateral inguinal herniorrhaphy. A previous study clearly indicated that the trauma involved in a unilateral herniorrhaphy under a spinal anesthesia is sufficient to cause a postoperative abnormality in glucose tolerance. It was considered, therefore, that the operative trauma in each case of the present study was sufficient stimulus to evoke changes in metabolism.

Results

The mean values (± standard error) demonstrating the effect of surgery on the fasting blood concentrations of carbohydrate intermediaries are listed in Table 1. These data, derived from 12 subjects before and 1 day after surgery, indicate there was a statistically significant rise in the blood concentration of glucose and of pyruvic and lactic acids, but no significant change in the blood concentration of citric acid.

Results demonstrating the reproducibility of the citrate-tolerance test, which was employed in this study, are recorded in Table 2.

When the citrate-tolerance tests were performed in 5 patients during and after surgery, no significant difference was found in

TABLE 2.—*Citrate Tolerance in 3 Successive Control Tests: Citric Acid, Milligrams per 100 Ml.**

		Time (Minutes)						
	Date	0	30	60	90	120	150	180
Control 1	12/23	2.7	6.4	8.3	4.6	3.5	3.3	3.4
	1/2	1.9	6.1	8.6	4.9	3.4	2.6	3.2
	1/9	2.1	7.4	8.6		3.9	2.9	2.8
Control 2	1/21	2.3	6.5	7.8	4.8	3.5	2.8	2.6
	1/28	1.7	6.9	7.9	3.9	2.9	2.5	2.2
	2/4	2.2	7.3	8.5	4.5	3.2	2.8	2.5

* A solution of 2½% sodium citrate given intravenously for 1 hour on the basis of 0.065 gm./kg.

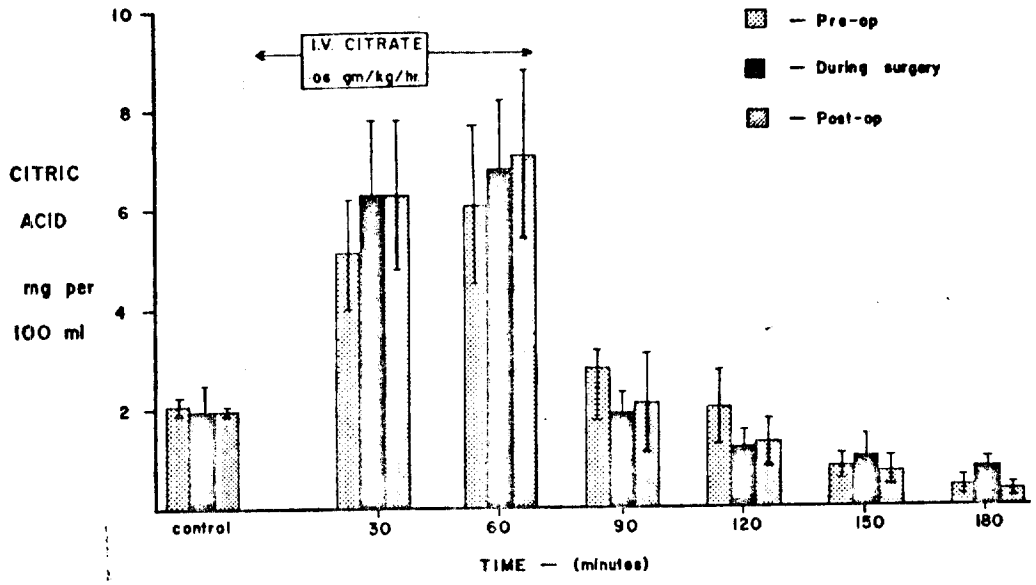

Fig. 1.—Effect of surgery on citrate metabolism (mean rise above control, 5 patients).

comparison with their control preoperative tests. The lack of statistical significance in these tests is illustrated in Figure 1 by the thin lines which represent the standard error of the mean for the 5 patients.

A statistically significant difference was found in the response of blood glucose to citrate infusion during surgery. In comparison to the preoperative study, the mean glucose levels were found to be significantly

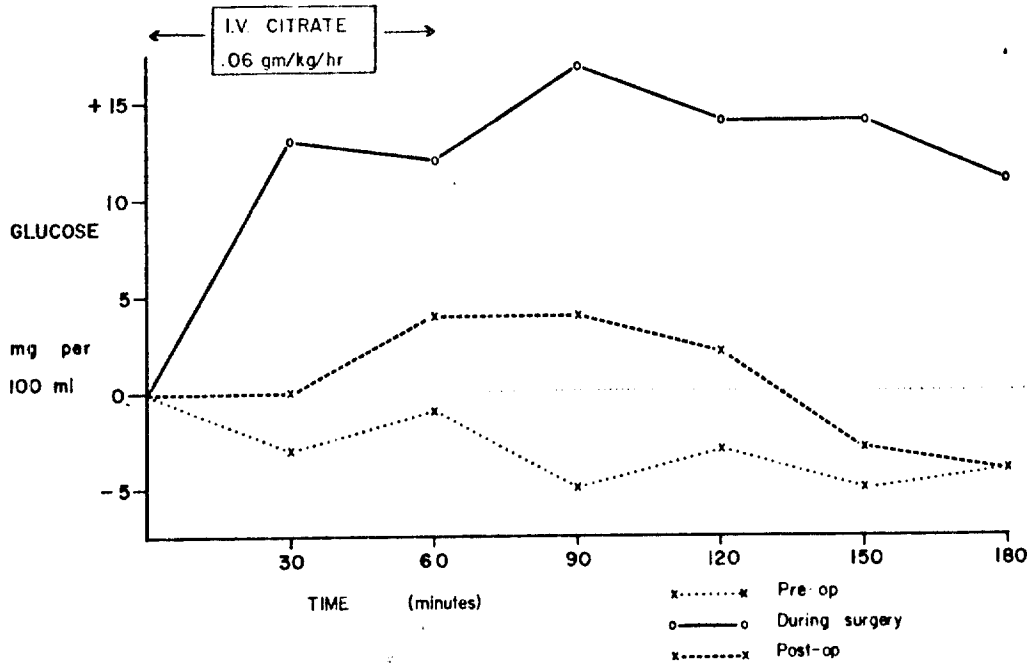

Fig. 2.—Blood glucose during citrate infusions (mean change from control, 5 patients)

TABLE 3.—*Blood Concentration of Carbohydrate Intermediaries During Citrate Tolerance Tests: (Mean Change from Preinfusion Concentration for 5 Subjects)* *

Time After Start of Infusion (Min)	Glucose			Pyruvic Acid			Lactic Acid *			Inorganic Phosphorus		
	Preop	Op.	Postop.	Preop.	Op.	Postop.	Preop.	Op.	Postop.	Preop	Op.	Postop.
30	−3 ‡	13	0	0.1	0.1	−0.3	−0.7	−0.6	0.3	0	0.2	−0.5
60	−1	12	4	0	0.3	−0.4	−0.9	−0.2	−1.0	−0.5	0.2	−0.1
90	−5 ‡	17	4	−0.1	0.2	−0.2	−1.6	−0.3	−1.5	−0.4	0	−0.2
120	−3	14	2	−0.1	0.1	−0.2	−1.9	−1.2	−1.8	−0.3	−0.3	−0.5
150	−7 ‡	11	−3	0	0.1	−0.2		−2.1	−2.4	−0.3	−0.5	−0.1
180	−4	9	−4	0	0	−0.2	−2.8		−2.0	−0.2	−0.5	−0.3

* 2½% sodium citrate given intravenously for 1 hour on the basis of 0.065 gm/kg.
† Mean values of 4 subjects.
‡ Comparison of preoperative with operative tests $p < 0.05$.

elevated 30, 90, and 150 minutes after the start of the citrate infusion on the day of surgery, $p<0.05$ (Fig. 2 and Table 3). On the day after surgery the glucose levels did not fall as rapidly with the infusion of citrate as in the preoperative tests, but the mean differences between the 2 tests were not statistically significant.

Lactic and pyruvic acids and serum inorganic phosphorus fell less with the administration of citrate during surgery than during the preoperative tests (Fig. 3). Although the changes were consistent, the mean differences from the pre- and postoperative tests were not statistically significant (Table 3).

Comment

During the past quarter century, it has become well established that citric acid metabolism is intimately related to carbohydrate metabolism in plants, animals, and man.[7] The rate-limiting step between the Embden-Myerhof anaerobic scheme of glycolysis and the Krebs tricarboxylic acid cycle

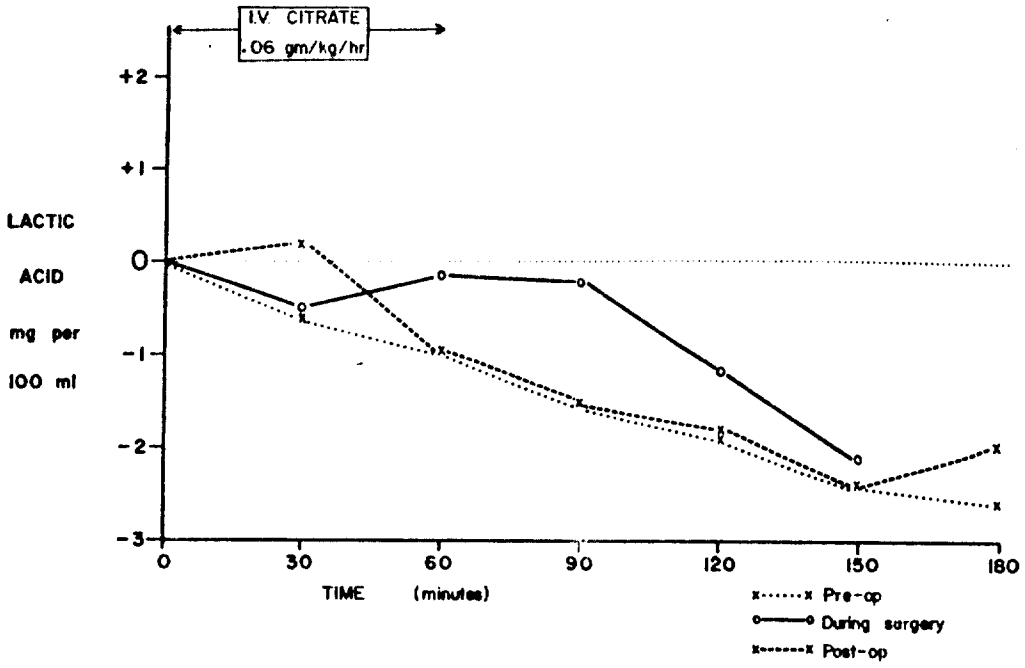

Fig. 3.—Lactic acid during citrate infusions (mean change from control, 5 patients).

is the availability of oxaloacetate (Fig. 4). Ordinarily, one 2-carbon molecule of acetyl-Co-A combines with a 4-carbon molecule of oxaloacetate to form a 6-carbon molecule of citrate. Subsequent oxidative reactions liberate 2 molecules of carbon dioxide and reform one 4-carbon molecule of oxaloacetate. Thus, for each cycle there is no net gain of oxaloacetate.

When an additional source of oxaloacetate is provided by the exogenous administration of citrate or acetate, additional quantities of acetyl-Co-A may be metabolized.[8] In turn, the flow of pyruvate to acetyl-Co-A, of lactate to pyruvate, as well as of glucose to pyruvate may be accelerated. The net result would be the fall observed in the concentrations of blood glucose and of pyruvic and lactic acids with the preoperative infusions of citrate (Figs. 2 and 3 and Table 3). The concomitant slight fall in serum inorganic phosphorus suggests that formation of high-energy organic phosphate compounds is also increased by the administration of citrate.

Due to the many alterations in the humoral environment that occur with surgery, the response to citrate infusion during surgery is more difficult to interpret. Failure to find a significant change in the rate of assimilation of exogenous citrate suggests that the tricarboxylic acid cycle is capable of normal function both during and after surgery (Fig. 1). But the increase in the fasting concentration of venous blood glucose and of lactic and pyruvic acids clearly indicates that surgery causes a disturbance in intermediary metabolism at a time when the metabolism of citrate is apparently normal (Table 1)—at least normal by the criteria of no alteration in citrate tolerance as well as no elevation in the fasting blood concentration of citric acid.

Similar changes in blood glucose and in pyruvic and lactic acids have been found in patients maintained on high doses of cortisone or in patients with Cushing's syndrome. Henneman and Bunker have ascribed these changes to inhibition of the conversion of pyruvate to acetyl-Co-A by the 17 hydroxycorticosteroids.[9] In view of the normal concentration of citrate and α-ketogluterate as well as a normal change in the concentration of both ketones and total fatty acids after glucose administration, they considered the tricarboxylic cycle to be unaffected by the high levels of 17 hydroxycorticosteroids.[9] Natelson's studies indicate that the metabolism of citrate is also independent of insulin action.[10]

Thus, insofar as integrity of citrate metabolism reflects normal function of the Krebs cycle, the results of the present study indicate that the reactions of the Krebs cycle are not inhibited by the postoperative altered humoral state (Fig. 1). The rise in blood glucose found when citrate was infused during surgery, as opposed to the fall in glucose with citrate administration preoperatively, may reflect an increased conversion of Krebs cycle constituents to glucose in the presence of impaired glucose assimilation (Fig. 4). The less marked rise in glucose with citrate administration on the first postoperative day in comparison with the test during surgery reflects a less severely disorganized metabolic state with less conversion of Krebs cycle constituents to glucose.

The decreased fall in lactate with citrate infusions during surgery may reflect inhibition of the conversion of pyruvate to acetyl-Co-A by 17 hydroxycorticosteroids as well as less available oxaloacetate to combine with acetyl-Co-A, due to the diversion of Krebs cycle intermediates, such as oxaloacetate to glucose (Figs. 3 and 4).

These results, in conjunction with the previous studies of glucose, fructose, lactate, and pyruvic tolerances, therefore suggest that 2 sites of metabolic inhibition occur after surgery (Fig. 4). One site probably involves the assimilation of glucose; the other involves the conversion of pyruvate to acetyl-Co-A. From a practical point of view, these results suggest that the intravenous administration of citrate would be useful for alimentation in the postoperative patient. The question of toxicity of citrate naturally arises, although no alterations were found in electrocardiogram or serum calcium or blood pH in the course of the present studies. Exceedingly high levels of citrate have been recorded in

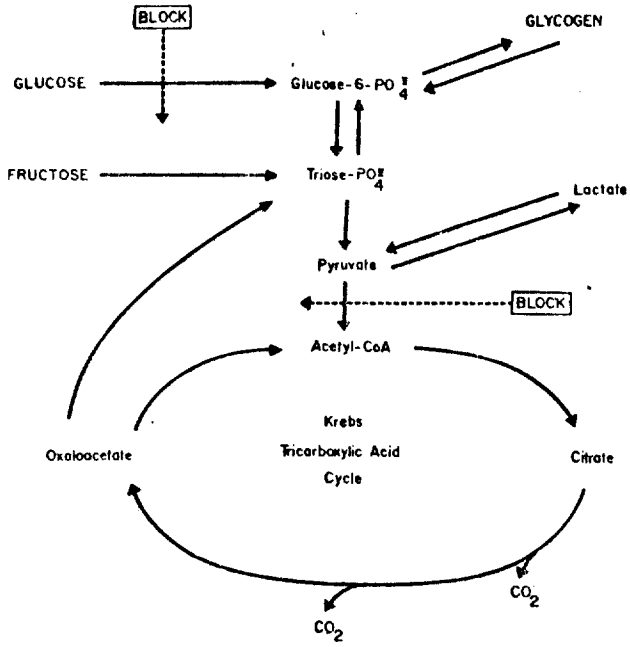

Fig. 4.—Proposed sites of metabolic inhibition produced by surgery.

patients who received large volumes of blood, and in some instances these very high levels have been thought to contribute to the death of the patient.[11] Others have not observed citrate toxicity despite the administration of large quantities of blood and the rise of citric acid to levels over 100 mg/100 ml.[12] Should citrate intoxication occur, it is primarily a reflection of a deficiency of ionized calcium. While the present studies indicate that surgery and anesthesia do not increase the possibility of citrate intoxication, it does seem reasonable to administer supplementary calcium if large quantities of citrate are given. This would be particularly true if further study indicates optimal postoperative alimentation would be achieved by the use of sodium citrate. Since most of the energy for body processes is derived from reactions in the Krebs citric acid cycle, and since there is no apparent alteration in the function of this cycle after surgery, a therapeutic use for citrate may be indicated.

Summary and Conclusions

1. The ability of man to assimilate exogenous citrate is not altered during or after surgery with general anesthesia.

2. On the basis of the present work and previous studies with glucose, fructose, lactate, and pyruvate, it is suggested that surgery causes inhibition of 2 pathways in intermediary metabolism, but no impairment of the Krebs citric acid cycle.

3. Since most of the energy for body function is produced in the citric acid cycle, a possible therapeutic value of citrate is considered.

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Food Protection Committee, 1972

Comprehensive GRAS Survey, daily intakes — citric acid

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table 13, Part A, p. 65

Food Protection Committee, 1972

Comprehensive GRAS Survey, Annual Poundage data -- citric acid

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table 11, Part A, p. 4

Food Protection Committee, Food and Nutrition Board

Chemicals Used in Food Processing

National Academy of Sciences, National Research Council, Washington, D.C.
Publication 1274, pp. 15, 43, 101, 264

Furia, T.E., 1972

CRC Handbook of Food Additives, 2nd edition

Chemical Rubber Co., Cleveland, Ohio
Pp. 244-245

J. Pharmacol. Exptl. Therap. 94: 65-67

A STUDY ON THE COMPARATIVE TOXIC EFFECTS OF CITRIC ACID AND ITS SODIUM SALTS

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Since the introduction of citrated blood for transfusion in 1915 by Lewisohn (1), there has been considerable study as to its value and its disadvantages. In the original work, the relative safety of the procedure was demonstrated first in dogs and then in man. Following this, much was done to improve the technique and thereby reduce the number and severity of the untoward reactions. In recent years under the stimulus of massive transfusions of citrated blood for treatment of extreme shock produced by hemorrhage, there has been much study and speculation as to the occurrence of citrate intoxication (2). However, there apparently have been no comparative studies as to the toxicity of citric acid and its sodium salts as measured by the LD_{50}, although anticoagulant acid citrate dextrose solution is U.S.P. The following work was undertaken to determine the LD_{50} for citric acid as well as for its sodium salts.

METHOD. White mice, albino rats, rabbits and dogs were used as experimental animals. Equal molecular concentrations of citrate in the forms of citric acid, monosodium citrate, disodium citrate, and trisodium citrate were compared. Five hundred and ninety-nine intraperitoneal injections of 0.0477 Molar solutions were made in four hundred and seventy-seven mice weighing between 14 and 28 grams with an average weight of 19 grams. Three hundred and eighty-four intraperitoneal injections of 0.381 Molar solutions were made in two hundred and ninety-nine rats weighing between 85 and 170 grams with an average weight of 120 grams. Three hundred and sixty-one intravenous injections of 0.0119 Molar solutions were made into the tail veins of two hundred and seventy-nine mice weighing between 14.5 and 28.5 grams with an average weight of 22 grams and in these the whole dose was given within a few seconds. One hundred and fifty-eight intravenous injections of 0.477 Molar solutions were made into the lateral ear veins of one hundred rabbits weighing between 1.75 and 2.75 kilograms with an average weight of 1.95 kilograms. Here a constant injection rate of 0.75 cc./minute (0.358 millimoles/minute) was used. The LD_{50} was determined with each method of administration for citric acid, monosodium citrate, disodium citrate, and trisodium citrate by the method of Reed and Muench (3).

Eighty intravenous injections of 0.25 Molar solutions were made into the tail veins of eighty mice weighing between 19 and 29 grams with an average weight of 24 grams. Twenty mice were used for each compound. In these experiments the injections were made at a constant rate of 6 cc./minute and continued until the animals died. The individual lethal doses in millimoles per gram of each of the citrate solutions injected were averaged for each twenty animals, and the standard deviations determined.

Under ether anesthesia lumbar cordotomies were performed on seven dogs weighing between 10 and 15 kgm. As soon as the animals recovered from the anesthetic the left femoral artery was cannulated and connected to a mercury manometer using heparin as the anticoagulant. Four animals received trisodium citrate (2.94 grams/kilogram/100 cc.) and the other three were given equal molar quantities of citric acid (2.10 grams/kilogram/100 cc.) intravenously at a constant rate of 0.67 cc./minute until death resulted.

RESULTS. The visible responses of mice, rats, rabbits and dogs to toxic doses of citric acid, monosodium citrate, disodium citrate, and trisodium citrate were similar and consisted primarily of increased general activity, hyperpnea, vasodilatation of the peripheral vessels, salivation, muscle twitching, clonic and tonic convulsions, cyanosis, Cheyne-Stokes respiration and some deaths. In all of the animals receiving single injections except those injected intraperitoneally with citric acid, if recovery occurred it was apparently complete within a few minutes. These findings are explained adequately by the fact that there is formation of double salts with calcium which do not liberate calcium ions (4).

Since this is intended as a comparative study the doses are expressed in millimoles/kilogram in table I instead of the usual grams/kilogram. Because the molecular concentration of the citrate ion was kept constant, in so far as citrate ion effect is concerned, the LD_{50} determinations of the various salts can be compared directly. Citric acid and its sodium salts have the same toxicity when given slowly intravenously to rabbits (see table 1). When citric acid was given

TABLE I

The toxicity of citric acid and its sodium salts in millimoles/kilogram as measured by means of the LD_{50}

ANIMAL	NO.	ROUTE OF ADMINISTRATION	CITRIC ACID	MONO-SODIUM CITRATE	DI-SODIUM CITRATE	TRI-SODIUM CITRATE
Rabbits	158	Intravenous at rate of 0.358 millimoles/minute	1.72	1.76	1.77	1.74
White mice	361	Rapid intravenous	0.22	0.23	0.30	0.66
White mice	599	Intraperitoneally	5.0	7.6	7.5	5.5
Albino rats	384	Intraperitoneally	4.6	6.3	7.3	6.0

intraperitoneally to rats and mice a number of animals died as long as one week after recovery from the immediate toxic effects. Gross post-mortem examinations performed upon many of these animals did not reveal the cause of death. If these compounds are injected rapidly intravenously in mice significant differences in the toxicity are observed (see table I). It would appear that the acid rather than the citrate part of the molecule is the cause of this difference in toxicity.

In those experiments on mice in which intravenous injections of 0.25 Molar solutions were made at a constant rate of six cubic centimeters per minute (1.5 millimoles of the drug per minute) no significant differences in the averages of the individual lethal doses were noted. In the 80 experiments (20 for citric acid and 20 for each of its sodium salts) the average dose necessary to kill all of the animals with the standard deviation was 2.08 ± 0.11, 2.01 ± 0.09, 2.21 ± 0.10, and 2.24 ± 0.51 for citric acid, monosodium citrate, disodium citrate and trisodium citrate respectively.

In the experiments on dogs in which blood pressures were recorded there was a gradual fall in blood pressure during citric acid injection until near death when the pressure fell precipitously to zero. When sodium citrate was used the blood

pressure remained fairly normal during the injection until just prior to the death of the animal when it fell abruptly to zero.

DISCUSSION. There are four criticisms to drawing any conclusions as to the effect of citrated blood for transfusion purposes in man from our results: first, our results were obtained from animals and may not be directly applicable to man on a kgm./kgm. basis; second, these animals were injected with pure drug, whereas, the citrate solution may be altered by the presence of the blood for transfusion; third, the rate of injection in these animals was much more rapid in millimoles/kgm./minute than would ever be given to man in the form of citrated blood; fourth, the patients are not well at the time of transfusion.

Theoretically, ignoring the above criticisms and assuming the LD_{50} for man to be about 1.75 millimoles/kgm., or near that for the slow intravenous injection of rabbits, about one liter of anticoagulant acid citrate dextrose solution U.S.P. would have to be given to a 70 kgm. man in a 15 minute interval or less in order to cause the demise of one-half of the patients so treated.

SUMMARY

1. The train of symptoms following the administration of citric acid and its sodium salts in toxic quantities appears to be identical with that of calcium ion deficiency consisting of increased general activity, hyperpnea, vaso-dilatation of the peripheral vessels, salivation, muscle twitching, clonic and tonic convulsions, cyanosis, Cheyne-Stokes respirations and some deaths.

2. The LD_{50} for citric acid, monosodium citrate, disodium citrate and trisodium citrate in millimoles/kgm was as follows: intravenous administration in rabbits 1.72, 1.76, 1.77, and 1.74 respectively; for intravenous administration in mice 0.22, 0.23, 0.30, and 0.66 respectively; for intraperitoneal administration in mice 5.0, 7.6, 7.5 and 5.5 respectively; and for intraperitoneal administration in rats 4.6, 6.3, 7.3, and 6.0 respectively. These results suggest that citrate intoxication in massive transfusions does not occur.

We wish to thank Dr. Charles M. Gruber and Dr. Harold W. Jones for their assistance.

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FOOD ADDITIVES

Safety of Adipic Acid as Compared with Citric and Tartaric Acid

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Increased usage of adipic acid as a food additive has prompted the comparison of it with citric and tartaric acids. Acute and chronic administration to laboratory animals has shown that adipic acid is comparable to these acids and is a safe food additive.

ADIPIC ACID (1,4-butanedicarboxylic acid), citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid), and tartaric acid (1,2-hydroxy-1,2-ethanedicarboxylic acid) are straight chain organic acids of 6, 5, and 4 carbon atoms, respectively. Adipic acid has no substituted groups, citric acid has both a hydroxy and carboxylic group substituted on the second carbon, while tartaric acid has a substituted hydroxy group on both the first and second carbons. Of these acids, only adipic is nonhygroscopic. Because of the increased interest in their use as food additives, the following work was undertaken.

Review of Available Literature

Rose (*10, 11*) is responsible for several investigations in the course of which he found that, following subcutaneous administration, adipic acid was mildly irritating to the kidneys, while glutaric acid (1,3-propanedicarboxylic acid) was nephrotoxic. In 1925, Rose and coworkers (*12*) concluded from their investigations that none of the higher homologs were irritating to the kidneys. Corley and Rose (*1*) examined 19 different acids for nephrotoxicity but found that only three—tartaric, mucic, and glutaric—exerted pronounced toxicity. Mucic acid is tetrahydroxyadipic acid. These authors concluded that: The number of carbon atoms, per se, present in a dicarboxylic acid has no relation to its toxicity; the introduction of a hydroxy or ketonic group on the first carbon of glutaric acid destroys its nephrotoxic effects; and the introduction of a hydroxy or ketonic group on the first carbon of adipic acid does not influence the toxicity.

Somewhat later, Harding and Nicholson (*8*) directed studies toward evaluating this apparent discrepancy in toxicity in acids which possessed rather similar properties. Their studies indicated that following either subcutaneous or intraperitoneal administration, citric acid was readily absorbed with a minimum of local reaction, whereas adipic acid caused marked local reaction at the site of injection and higher homologs of the series could be definitely identified as retained crystals at the site. Based on these observations, at least a portion of the difference in renal toxicity might be due to poor absorption of adipic acid and the higher homologs; their work confirmed the nephrotoxicity of glutaric acid. However, following subcutaneous administration, Flaschenträger (*4*) recovered approximately 50% of administered adipic acid in the urine.

In 1942, Enders (*2*) reported that adipic acid, azelaic acid (1,7-heptane dicarboxylic acid), and sebacic acid (1,8-octanedicarboxylic acid) are only slightly toxic when given in large single oral doses to rabbits, or when fed daily to rats over a long period of time. Excretion of these acids in the urine of rats showed adipic acid to be more slowly excreted than the others.

Simola and Kosunen (*13*) fed the sodium salts of a series of organic acids in single doses to adult rats and analyzed the urine for increased citric and ketonic acid excretion. All of the acids studied increased both the citric and ketonic acids, but the increase was slight with adipic acid.

Both Hanson (*7*) and Weitzel (*14*), in their studies on the urinary recovery of orally administered adipic acid to humans, concluded that decomposition took place in the body with small amounts being more completely catabolized than large amounts.

An unpublished report by Foulger (*5*) is of particular interest from a practical standpoint as it presents the results following the repeated administration of relatively large doses of adipic acid. Immature rats failed to gain weight properly when given 653 to 1332 mg. per kg. Fitzhugh and Nelson (*3*) reported 2-year rat feeding experiments on several acids, including tartaric acid, which they found was not toxic in concentrations up to 1.2% of the diet.

More recently, Gruber and Halbeisen (*6*) reported that, following intraperitoneal administration to rats, adipic acid appears to be more toxic than citric. They reported deaths from citric acid up to 1 week but could not associate this with postmortem findings. The intraperitoneal administration of adipic acid resulted in extensive irritation and adhesion of visceral organs. A rapid intravenous injection of citric acid in mice resulted in an LD_{50} of 0.22 millimoles per kg. When the injection was made at the rate of 1.5 millimoles of acid per minute until the animals died, the average LD_{100} was 2.08 millimoles per kg.

Experimental

Acute Oral Administration. Male albino mice were used in this study. A 3% aqueous solution of adipic acid, kept at body temperature, was tried but proved impractical as sufficiently large doses to determine an LD_{50} could not be administered. Therefore, a 6% suspension of adipic acid in 0.5% methyl cellulose was administered orally, resulting in an LD_{50} of 1900 mg. per kg. or 13.0 millimoles per kg. (Table I). Autopsy of the animals that died showed distention of the stomach and small intestine, with a spastic concentration of the caecum. Irritation and hemorrhage of the intestines were noted. Initial mortality developed overnight and deaths continued throughout the first week, after which survivors appeared normal. All animals were sacrificed after 10 days.

Acute Intraperitoneal Administration. A few mice were given lethal doses (600 and 900 mg. per kg.) of a 3% aqueous solution of adipic acid intraperitoneally. These mice showed depression immediately and, at autopsy, the intestines appeared irritated and the lungs appeared hemorrhagic.

Male albino rats were given a 3% aqueous solution of adipic acid intraperitoneally (Table I). Mortality occurred during the first 5 days. The LD_{50} was 2.75 mg. per kg. (1.88 millimoles per kg.). Animals that succumbed showed hemorrhagic lungs and irritation of the intestines. The survivors, sacrificed 1 week after administration, showed extensive irritation and adhesions of the visceral organs.

Acute Intravenous Administration. Intravenous injection to mice at various dosage levels, at a rate of 0.01 ml. per second, with 2% solutions of adipic,

[1] Deceased.

Table I. Acute Toxicity of Adipic, Citric, or Tartaric Acid to Male Albino Mice or Rats

(Dosages are as the acid. Values are the number of animals dead per number of animals tested)

Dose, Mg./Kg.	Adipic			Citric,	Tartaric,
	Oral[a], mice	Intraperitoneal[b], rats	Intravenous[c], mice	Intravenous[c], Mice	Intravenous[c], Mice
175				2/13	
200		1/7		6/13	0/3
225				10/13	
250				3/3	
300		4/7			
350		6/7			
400					0/2
450					1/13
475					2/13
500					9/13
650			4/13		
675			7/13		
700			8/13		
1500	3/13				
2000	8/13				
2500	9/13				
LD_{50}, mg./kg.	1900	275	680	203	485
Confidence limits, mg./kg.	1640–2200	193–392	653–708	190–217	462–509
LD_{50}, millimoles/kg.	13.0	1.88	4.65	1.07	3.23

[a] 6% suspension in 0.5% methyl cellulose.
[b] 3% aqueous solution.
[c] 2% aqueous solution.

Table II. Summary of Average Body Weights of Albino Rats

(Controls received the basal diet. Other animals received the basal diet containing the indicated percentage of the adipic acid or citric acid)

	Average Body Weight in Grams								
	Males							Females	
		Adipic Acid				Citric Acid			Adipic acid,
Week	Control	0.1%	1%	3%	5%	3%	5%	Control	1%
0	59	61	63	61	57	62	61	49	48
8	269	280	265	224	182	239	225	178	175
16	325	333	320	276	233	298	278	222	213
24	361	374	354	309	264	329	320	242	233
32	377	391	376	329	291	328	339	257	249
40	397	407	401	357	314	370	361	279	263
48	423	433	421	372	322	393	377	275	270
56	428	447	436	380	336	400	388	286	277
64	426	455	436	385	339	407	401	295	284
72	407	447	431	385	336	400	389	301	288
80	408	441	430	383	349	411	391	313	301
88	413	448	432	398	344	411	389	309	303
96	432	424	436	396	354	409	393	318	308
104	440	417	437	400	360	417	397	321	304

Table III. Summary of Data for Albino Rats Receiving Basal Laboratory Diet or Basal Diet of Adipic or Citric Acid for 2 Years

(Per cent of survival based on length of survival as well as number of animals)

Level	Sex	No. of Rats		Av. Body Weight, G.		Food Consumed, G.,	Compound Consumed, Mg.,	Survival,
		Start	Finish	Initial	Final	Av./Rat/Day	Av./Rat/Day	%
Control	M	20	8	59	440	16.8		82.5
	F	10	8	49	321	14.2		98.9
Adipic acid								
0.1%	M	20	13	61	417	17.0	17.0	87.7
1%	M	20	15	63	437	17.5	175	94.7
	F	19	17	48	304	15.8	158	96.3
3%	M	20	16	61	400	16.8	505	94.5
5%	M	20	15	57	360	15.8	814	97.2
Citric acid								
3%	M	20	14	62	417	17.1	512	92.6
5%	M	20	16	61	397	15.7	784	95.0

citric, or tartaric acid yielded LD_{50} values of 680, 203, and 485 mg. per kg., respectively (4.65, 1.04, and 3.23 millimoles per kg.). The results of these experiments are presented in Table I. These acids caused immediate, convulsive deaths, probably due to acute acidosis as the pH of the solutions was 3.08, 2.50, and 2.53, respectively. Autopsy showed hemorrhagic lungs but no other gross pathology. In survivors, recovery was apparently complete and there were no latent deaths. Statistical analysis was done by the method of Litchfield and Wilcoxon (9).

Chronic Feeding. Young male and female albino rats of the Carworth Farms strain, having approximate mean initial weights of 60 and 50 grams, respectively, were selected at random for use in these studies. All of the rats were housed individually in cages with wire mesh floors elevated above the droppings. The animals had free access to food and water at all times.

Groups of rats were placed on either the basal laboratory diet or the basal diet containing either adipic acid or citric acid, as follows:

Group	Males	Females
Basal laboratory diet used as control	20	10
Basal diet containing 0.1% adipic acid	20	0
Basal diet containing 1% adipic acid	20	19
Basal diet containing 3% adipic acid	20	0
Basal diet containing 5% adipic acid	20	0
Basal diet containing 3% citric acid	20	0
Basal diet containing 5% citric acid	20	0

The body weights and food consumption of all rats were recorded at weekly intervals during the course of the study. In addition, weekly observations were made of the general appearance and condition of each animal. Whenever possible, gross autopsy was performed on those animals that died during the course of the experiment.

After 2 years on the respective diets, the surviving rats were weighed, sacrificed by a blow on the head, and examined for gross and microscopic pathology. The brain, thyroid, lungs, heart, liver, spleen, kidneys, adrenals, stomach, and testes of approximately half of each group of males were weighed. The kidneys, spleen, liver, and heart of each female were weighed. Microscopic examination of the following tissues were done on a representative number of animals of each group: thyroid, lungs, heart, liver, spleen, kidneys, adrenals, stomach, small intestine, large intestine, pancreas, bone marrow, testes or ovaries, and uterus.

Results

Males. The average body weights for the male rats are tabulated in Table II for each 8-week interval. Throughout the entire 2-year study, the 0.1 and 1% adipic acid groups were comparable with the control groups. During the rapid growth period, the weight gains of the 3 and 5% adipic acid and the 3 and 5% citric acid groups were significantly less than the control groups; however, there was no significant difference among these four test groups. Throughout the latter half of the study, the average body weights of the various test groups were not remarkable—although the 5% adipic acid group was consistently the lowest.

Table III presents a summary of food and compound consumed and survival data for the entire 2-year feeding period. There was only a slight, but consistent, reduction in food consumption by the 5% adipic acid and 5% citric acid groups. Other test groups were comparable to the control group. The per cent survival for each test group was better than the control group.

Autopsy data for the male animals that died during the course of the 2-year feeding program and for the sacrificed rats were analyzed for incidence of tumors and/or lung pathology. To be included in the following table, a tumor must have presented gross evidence of being a new growth.

Females. The average body weights for the female rats are tabulated in Table II for each 8-week interval while food and compound consumption, together with survival data, are presented in Table III. There was no significant difference between the body weight gains or food consumption for the two groups.

In the last 6 months, the animals exhibited signs normally associated with advancing senility in rats. There was an equal incidence of blood-tinged crust about the eyes and noses, unthriftiness, and body sores in both groups. A few control and experimental animals had alopecia, and one experimental rat appeared to develop a middle ear infection during the 102nd week. The average weight of the kidneys, spleen, liver, and heart, together with organ-to-body weight ratios, appeared to be within normal limits.

One experimental and two control animals died during the final 6 months.

	Deaths				Sacrificed	
Male Group	Lung pathology	Tumors	Other causes	Total deaths	Lung pathology	Tumors
Control	7	3	3	12	4	1
Adipic						
0.1%	3	2	3	7	7	2
1%	1	2	2	5	7	2
3%	3	..	1	4	3	..
5%	..	4	1	5	4	..
Citric						
3%	1	2	3	6	1	..
5%	1	2	1	4	4	1

These findings appear not to be related to the compounds under study as an equivalent incidence was observed in the controls.

Throughout the study, especially the final 6 months, the following signs were observed among all the groups, including the controls: wheezing, blood-tinged crust about the noses and eyes, and body sores. The incidence of these findings did not appear to be significantly different among the groups although a lower incidence of signs indicative of respiratory infection and body sores occurred in the 5% adipic acid group.

When the surviving males were sacrificed at the end of the 2-year period, there was no significant gross pathology that could be related to either compound. Soft edematous testes were noted at least as frequently in the controls as in the experimental animals. There was no significant difference in organ weights of the experimental groups *vs.* the controls.

Microscopic examination of thyroid, lungs, heart, liver, spleen, kidneys, adrenals, stomach, pancreas, bone marrow, and large and small intestines revealed these tissues to be within normal limits in all groups of male rats.

All three exhibited diarrhea, respiratory infection, and loss of weight prior to death. Upon autopsy, one control rat and one experimental rat were found to have tumors, while the other control animal had a granular liver and dark red apexes on both lungs.

When the surviving animals were sacrificed at the end of the 2-year period, there was no significant gross pathology that could be related to ingestion of the compound. There was an equal incidence of mottled, granular livers with peripheral thickening in both the control and experimental animals. Two of the surviving control rats and one experimental animal had ovarian tumors; ovarian cysts were noted in both control and experimental rats.

Discussion

The results of the above experiments indicate that adipic acid is significantly less toxic than tartaric or citric acid following intravenous administration to mice. The doses were calculated as milligrams per kg. and as millimoles per kg. The action of these three acids appears comparable.

No direct comparison of the intravenous toxicity of citric acid in these tests and those reported by Gruber and Halbeisen (*6*) is possible because they used a faster rate of injection. The LD_{50} values of 1.06 millimoles per kg. obtained in this experiment is midway between the LD_{50} and the LD_{100} of Gruber and Halbeisen.

Single oral administrations of an almost saturated solution (3%) of adipic acid did not cause appreciable mortality in tolerable volumes. With a 6% suspension, the LD_{50} approximated 2 grams per kg. Comparable values for citric and tartaric acids are not available.

Following intraperitoneal administration to rats, adipic acid appears to be more toxic than citric (*6*). The intraperitoneal administration of adipic acid resulted in extensive irritation and adhesion of visceral organs.

During the rapid growth period of the 2-year feeding studies, weight gains for the male rats receiving 3 or 5% adipic or citric acid was significantly less than the male controls; however, there was no significant difference among these four experimental groups. Growth for other groups—0.1 and 1.0% male and 1.0% female—was comparable to that of the respective controls. There was no evidence of gross pathology associated with the feeding of either acid. There was no significant difference in survival among the various groups from the controls. The incidence of lung pathology, tumors, or soft testes was observed at least as frequently in the controls. The organ-to-body weight ratios appeared to be within normal range. The results of microscopic examination appeared to be within normal limits for the representative tissues studied.

Comparison of the chronic feeding of adipic acid with citric acid (herein reported) and also with tartaric acid in an equivalent study (*3*) indicates that adipic acid is comparable with citric and tartaric acids.

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FURTHER OBSERVATIONS ON THE TERATOGENIC NATURE OF INSULIN AND ITS MODIFICATION BY SUPPLEMENTARY TREATMENT

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It was shown previously that insulin treatment of developing chicken embryos produces morphological abnormalities. These defects are chiefly, perhaps exclusively, skeletal, but what parts of the skeleton are affected depends on the stage during which the embryos had been exposed to the hormone. Incidence and degree of malformation vary with dose of injected insulin, hereditary constitution of the embryos, season, and probably numerous other and minor factors. Very high doses of insulin are sometimes responsible for the simultaneous occurrence of abnormalities which with smaller amounts only occur after treatment in other developmental stages. The fact that all insulin-induced malformations appear to be phenocopies of known mutants adds greatly to the interest of analyzing the physiological events by which insulin produces its effects. The more important results of our experiments have been reviewed in an earlier publication (Landauer, '48a). Since then we have found that the teratogenic consequences of insulin injection can be averted, in part or entirely, by supplementing insulin treatment with certain other substances, such as nicotinamide and α-ketoglutaric acid (Landauer, '48b). The present report deals with an extension of these experiments.

Nicotinamide

The injection of insulin into the yolk sac of chicken eggs during the 4th or 5th day of development is followed by a high

incidence of micromelia and beak defects. From the results of earlier experiments it appeared that the frequency of occurrence of micromelia could be much reduced by supplying nicotinamide along with insulin, but that such supplementation was less effective in regard to beak development. Survival of insulin-nicotinamide treated embryos was, however, less good in these experiments than in the group that had only been exposed to insulin, and, because of this, some doubt remained concerning the validity of the conclusions drawn from these data. Hence, we decided to repeat the experiments. At the same time we desired to obtain information on the following two questions. Does nicotinamide actually protect the embryos less well against insulin-induced beak defects than against abnormalities of the extremities? Secondly, will nicotinamide supplementation forestall the phenotypic effects that insulin has on hereditary polydactylism (Landauer, '48c)?

For our new experiments on the combined use of insulin (Iletin, Lilly) and nicotinamide (Niacinamide, Squibb) we used eggs from a mating of White Leghorn females to a Dorking male, the latter being homozygous for polydactylism. The results for incidence of micromelia and beak defects are given in table 1. Each of the three experimental groups included one lot in which insulin alone, in a dose of either two units (A and B) or one unit (C), was injected at 96 hours of development and another lot that served as untreated control. In addition, one lot of group A received two units of insulin and 5 mg nicotinamide at 96 hours, one of group B was given 10 mg of nicotinamide at 93 hours, followed by two units insulin at 96 hours, and one lot of group C was injected with 10 mg nicotinamide at 93 hours and one unit insulin at 96 hours. The results are unequivocal. When 5 mg nicotinamide were given simultaneously with two units insulin, the incidence of all types of malformations was much reduced; when the embryos were supplied with 10 mg of nicotinamide three hours prior to the injection of either one or two units of insulin, protection against the teratogenic effects of insulin was complete. In none of the three groups had nicotinamide a statistically significant effect on embryo mortality due to injection of insulin. In other tests, the results of which are not reproduced here, we found that higher doses of nicotinamide (20 mg) — similar in amount to those

TABLE 1

Experiments concerning the effect of nicotinamide on insulin-induced abnormalities of extremities, beak and eyes

F_1 embryos from Leghorn ♀♀ × Dorking ♂

GROUP	TREATMENT	MORTALITY PRIOR TO 18TH DAY	SURVIVORS OF 17TH DAY	MICROMELIA	BEAK ABNORMALITIES	EYE ABNORMALITIES
		%		%	%	%
A	Insulin 2 U. — 96 hours	26.8	60	48.3	33.3	6.7
	Insulin 2 U. + 5 mg nicotinamide — 96 hours	13.6	70	10.0	4.3	1.4
	Untreated	3.7	79	0	0	0
B	Insulin 2 U. — 96 hours	12.9	74	55.4	29.7	1.4
	Nicotinamide 10 mg — 93 hours, insulin 2 U. — 96 hours	19.5	66	1.5	0	0
	Untreated	10.0	72	1.4	0	0
C	Insulin 1 U. — 96 hours	24.4	96	16.7	9.4	2.1
	Nicotinamide 10 mg — 93 hours, insulin 1 U. — 96 hours	29.1	100	0	1.0	0
	Untreated	10.7	117	0	0	0

used in our earlier experiments — were definitely toxic whether used in conjunction with insulin or alone, but among 33 survivors only a few showed micromelia and none had beak defects.

It is clear from these results that the occurrence of those effects of insulin treatment (during the 4th or 5th day of incubation) which resemble the phenotypes of major gene

TABLE 2

Experiments concerning the effect of nicotinamide on insulin-induced modifications of polydactylism[1]

(This is the same material as in table 1)

F_1 embryos from Leghorn ♀♀ × Dorking ♂

TREATMENT	SURVIVORS OF 17TH DAY	A NON-POLY-DACTYLOUS	B BILATERAL POLY-DACTYLISM	C TYPICAL 5-TOED CONDITION ON BOTH FEET	D ATYPICAL BILATERAL POLY-DACTYLISM	E BILATERAL POLY-PHALANGISM	F HETERO-DACTYLISM
		%	%	%	%	%	%
Insulin 2 U. — 96 hours	60	58.3	33.3	18.3	15.0	1.7	6.7
Insulin 2 U. — 5 mg nicotinamide — 96 hours	70	55.7	28.6	17.1	11.5	4.3	11.4
Untreated	79	12.7	77.2	62.0	15.2	2.5	7.6
Insulin 2 U. — 96 hours	74	62.2	28.4	14.9	13.5	1.4	8.1
Nicotinamide 10 mg — 93 hours, insulin 2 U. — 96 hours	66	33.3	45.5	28.8	16.7	6.1	15.2
Untreated	72	11.1	76.4	62.5	13.9	4.2	8.3
Insulin 1 U. — 96 hours	96	40.6	40.6	25.0	15.6	5.2	13.5
Nicotinamide 10 mg — 93 hours, insulin 1 U. — 96 hours	100	20.0	46.0	33.0	13.0	11.0	23.0
Untreated	117	11.1	72.6	63.2	9.4	6.8	9.4

[1] Columns A, B, E and F contain the total of all observations; the data of B are broken down in C and D.

changes, viz. micromelia and beak defects, can be prevented by adequate and properly timed supplementation with nicotinamide. We have reported earlier that in polydactylous stocks insulin has modifier-like effects in regard to development of the toes. Data on the question whether or not these effects can be prevented by nicotinamide supplementation appear in table 2. These observations must be reviewed with reference to the different modes of expression of polydactylism. In the first place, it will be seen that, as reported earlier, insulin treatment greatly exaggerated the incidence of phenotypic lack of expression of the gene for polydactylism (column A). This insulin effect was not significantly altered when 5 mg nicotinamide were given together with two units insulin. When, however, 10 mg nicotinamide were injected three hours prior to either one or two units insulin the occurrence of non-polydactylous embryos was reduced very significantly ($\chi^2 = 9.90$ with $P = .002$ in the first instance and $\chi^2 = 11.71$ with $P = < .001$ in the second). As for the different modes of expression of polydactylism, it can be seen that under the influence of nicotinamide-supplementation there was a definite trend toward an increased incidence of the irregular types, such as heterodactylism and polyphalangism (E and F), in preference to a return to the bilaterally 5-toed condition which is typical for the control embryos. With optimal amounts of nicotinamide, however, the incidence of typical bilateral polydactylism was also augmented conspicuously (C). It is clear, therefore, that the changes which insulin produces in the expression of polydactylism can to some extent be prevented by nicotinamide-supplementation, but that the protective effect is lower than with reference to micromelia and beak abnormalities. This response difference will be discussed later.

In an earlier publication (Landauer, '48b) we have reported that a significant reduction in incidence of rumplessness was obtained when injection of two units insulin at 24 hours was supplemented with 18.9 mg nicotinamide, but that nicotinamide did not at this stage give the embryos as much

protection against insulin damage as it does at later stages (96 and 120 hours). Since the amount of nicotinamide used in these experiments proved to have considerable toxicity, we decided to repeat the experiment with a smaller amount of nicotinamide, viz. 5 mg. The results are summarized in table 3. The nicotinamide was injected three hours prior to insulin, simultaneously with it, or 3, 6 and 12 hours after insulin. Embryo mortality was, even with this lower dose of nicotinamide, much increased. When nicotinamide was given between three hours prior to and 6 hours following insulin treatment, the incidence of rumplessness was in each instance lower than with insulin alone. Corresponding to the smaller dose of nicotinamide, its effectiveness in forestalling rumplessness was reduced, and the differences were in no case great enough to be statistically significant. However, the χ^2 from the combination of probabilities for groups 2 to 5 as against group 1 was highly significant ($\chi^2 = 19.930$, df = 8, P = .01). Nicotinamide given 12 hours later than insulin no longer affected the incidence of rumplessness. On the whole, these results confirm with a lower dosage (viz. 5 mg) our earlier conclusion that an amount of nicotinamide which completely or nearly completely forestalled the micromelia-inducing effects of two units insulin at 96 or 120 hours of incubation, reduced only slightly the incidence of rumplessness after two units insulin at 24 hours and that the toxicity of this amount of nicotinamide was much greater at the earlier period than at the later one.

TABLE 3

Experiments concerning the effect of nicotinamide on the occurrence of insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 5 mg nicotinamide

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS (ALL TYPES)	ABNORMAL BEAK
			%		%	%
1	Insulin — 24 hours	200	30.5	121	26.4	0
2	Nicotinamide — 24 hours insulin — 27 hours	316	54.4	127	18.1	3.1
3	Insulin and nicotinamide 24 hours	347	62.8	119	21.0	5.9
4	Insulin — 24 hours nicotinamide — 27 hours	343	68.5	90	15.6	5.6
5	Insulin — 24 hours nicotinamide — 30 hours	359	54.3	138	15.2	3.6
6	Insulin — 24 hours nicotinamide — 36 hours	333	54.4	132	28.8	8.3

Some observations on beak defects, however, do not seem to fit into the general interpretation of the protective action of nicotinamide. In experiments with sulfanilamide, Zwilling and DeBell ('50) found that nicotinamide-supplementation completely forestalled the appearance of micromelia. But the parrot-beak condition that generally accompanies sulfanilamide-induced micromelia was, in the presence of nicotinamide, frequently replaced by a shortening of the upper beak. This effect of nicotinamide-supplementation was found when both sulfanilamide and nicotinamide were given at the end of the 5th day of incubation, but also when sulfanilamide was injected at 120 hours and nicotinamide 24 or even 48 hours later. We had observed earlier (Landauer, '48b) that nicotinamide supplementation of insulin treatment at 24 hours was responsible for the appearance of a certain proportion of embryos with shortened upper beaks. Our present results bear out these observations. We had suggested earlier that "a shift of the insulin damage within embryos" had been produced by nicotinamide. This interpretation is no longer tenable since it was found that nicotinamide by itself will in certain developmental stages call forth abnormalities of the beak. This is true for 24-hour embryos, but does not hold at all or to only a very slight extent at 96 hours (table 4). The observations of Zwilling and DeBell, to which we referred, suggest, however, that responses similar to those at 24 hours may occur again after 5 to 7 days of development.

Glucose-1-phosphate, citric and oxalacetic acid

Experiments in which insulin treatment at 96 hours was combined with the injection of glucose-1-phosphate produced results of considerable interest (table 5). Glucose-1-phosphate by itself was non-toxic and without teratogenic effects. When the injection of two units of insulin was supplemented with 4 mg glucose-1-phosphate the post-operative mortality was higher than after insulin alone; there was no significant

TABLE 4

Effect of nicotinamide, injected at 24 or 96 hours of incubation, on rate of mortality and beak development. Eggs from White Leghorn fowl

		AGE AT INJECTION 24 HOURS DOSE 5 MG	AGE AT INJECTION 96 HOURS		SIGNIFICANCE OF DIFFERENCES BETWEEN INJECTIONS AT 24 AND 96 HOURS FOR DOSE OF 5 MG
			Dose 5 mg	Dose 10 mg	
Number of fertile eggs injected		292	128	123	
Mortality during first 6 days and from 96 hours to end of 2nd week, respectively	%	16.1	3.1	8.1	$\chi^2 = 13.949$ $P = <.001$
Survivors of 17th day		235	117	110	
Short upper beak	%	7.2	0.9	1.8	$\chi^2 = 5.336$ $P = <.02$
Other abnormalities		0	0	0	

change in the incidence of micromelia or syndactylism (the latter not shown in the table) among the embryos surviving the 17th day of development, but the frequency of beak defects was greatly reduced. The experiment was performed twice with similar results. It will be noted that the incidence of micromelia and of beak abnormalities was considerably lower in the second experiment than in the first. This was true whether insulin was used alone or in combination with glu-

TABLE 5

The effect of supplementation of insulin-treatment with glucose-1-phosphate, citric acid and oxalacetic acid at 96 hours of incubation. White Leghorn eggs

Dosage of insulin 2 units throughout

DATE	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY FROM 96 HOURS TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICROMELIA	ABNORMAL BEAK	OTHER ABNORMALITIES	SIGNIFICANCE OF DIFFERENCES IN INCIDENCE OF BEAK ABNORMALITIES
			%		%	%	%	
4/25	Insulin + 4 mg glucose-1-phosphate	146	48.6	64	78.1	9.4	6.3	$\chi^2 = 7.006$ $P = <.01$
	Insulin	157	23.6	112	75.0	26.8	4.5	
5/30	Insulin + 4 mg glucose-1-phosphate	307	36.8	189	51.3	16.4	2.6	$\chi^2 = 15.678$ $P = <.0001$
	Insulin	153	28.8	96	49.0	37.5	5.2	
	Insulin + 25 mg citric acid	162	43.2	90	51.1	12.2	8.9	$\chi^2 = 15.607$ $P = <.0001$
	Insulin + 7.15 mg oxalacetic acid	150	42.0	83	37.3	14.5	2.4	$\chi^2 = 12.142$ $P = <.0001$

cose-1-phosphate and is an expression of a seasonal trend, already noted in earlier experiments (Landauer, '45). The relative reduction in frequency of beak defects under the influence of glucose-1-phosphate supplementation was, however, of a similar order in the two experiments. The beak abnormalities which occurred were of 4 different kinds, viz., parrot beak, shortened upper beak, cleft palate and crossbeak. Parrot beak is the only one of these 4 types of defects which, in its milder forms of expression, might lead to mistaken classification. It was, therefore, decided to scrutinize the effect of glucose-1-phosphate by adding all specimens with parrot beak to the normal groups, thus limiting the statistical analysis to those forms of abnormality in which we were dealing with presence or absence. Even with this, unquestionably over-stringent, procedure it was clear that glucose-1-phosphate furnished protection against insulin-induced beak abnormalities ($\chi^2 = 10.645$; $P = .001$).

A similar lowering in the incidence of beak defects was observed when insulin-treatment was supplemented with fructose-6-phosphate (5 mg/egg), but our data are too scanty to be trusted without reserve. Supplementation of insulin-treatment at 96 hours with fructose-1,6-diphosphate, on the other hand, clearly did not modify any of the teratogenic effects of insulin.

At 24 hours, supplementation of insulin with glucose-1-phosphate (4 mg/egg), fructose-6-phosphate (5 mg/egg) or fructose-1,6-diphosphate (2.7 mg/egg) had no significant effect on subsequent incidence of rumplessness. In two tests with glucose-1-phosphate we observed, however, that embryo mortality was increased significantly and micromelia was found in 10 and 11%, respectively, of the survivors of the 17th day. There were no cases of micromelia among the embryos treated with insulin alone.

Results similar to those with glucose-1-phosphate were obtained when insulin-treatment at 96 hours was combined with the injection of oxalacetic or citric acid. Embryo mortality was increased by these supplements, the frequency with which

micromelia occurred was not affected, but the incidence of beak defects was reduced to a highly significant extent (table 5). When given alone, oxalacetic acid had appreciable toxicity for 96-hour embryos (mortality to end of second week 24.1%), but citric acid was harmless.

At the 24-hour stage, supplementation with oxalacetic acid did not significantly change the incidence of insulin-induced rumplessness. Citric acid, on the other hand, greatly reduced embryo mortality and incidence of rumplessness when added to insulin treatment at 24 hours (table 6). The results are

TABLE 6

The effect of citric acid on insulin-induced rumplessness. White Leghorn embryos. All injections at 24 hours

Dosage: insulin 2 units, citric acid 25 mg

TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS (ALL TYPES)	OTHER ABNORMALITIES
		%		%	%
Insulin	165	52.1	65	29.2	9.3
Insulin + citric acid	171	17.0	135	11.1	2.9
Citric acid	165	3.6	157	1.3	0

highly significant in both respects (χ^2 for embryo mortality $= 46.035$, P .0001; for incidence of rumplessness $\chi^2 = 10.165$, $P = .0005$) and comparable to those obtained with lactic acid which will be discussed below. Isocitric acid (2.5 mg) given with insulin at 24 hours led to increased mortality and potentiation of the insulin effect (44.7% rumplessness, 10.5% beak abnormalities).

Pyruvic and lactic acid; sodium acetate

In testing for the alleviatory effect of other compounds on the teratogenic action of insulin we were particularly interested in relatively simple metabolic intermediates such as

pyruvic, lactic and acetic acid. Our observations with undiluted pyruvic acid (Matheson Co.) are summarized in tables 7 and 8. It can be seen that pyruvic acid, when given simultaneously with insulin, is very effective in forestalling the occurrence of rumplessness (table 7). The combined administration of these two substances at 24 hours of incubation reduced the incidence of rumplessness, in comparison to that after unsupplemented insulin treatment, from 23.1% to

TABLE 7

Experiments concerning the effect of pyruvic acid on insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ undiluted pyruvic acid

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS
			%		%
1	Insulin — 24 hours	254	45.3	130	23.1
2	Insulin and pyruvic acid — 24 hours	256	18.8	174	5.2
3	Insulin — 24 hours pyruvic acid — 27 hours	243	39.1	124	16.9
4	Insulin — 24 hours pyruvic acid — 30 hours	257	40.1	134	17.2
5	Insulin — 24 hours pyruvic acid — 36 hours	245	41.6	124	19.4
6	Pyruvic acid — 24 hours	171	10.5	146	2.7

5.2% ($\chi^2 = 21.252$; $P < .0001$), and embryonic mortality during the first 6 days dropped from 45.3% to 18.8% ($\chi^2 = 41.206$; $P < .0001$). If pyruvic acid is, however, supplied three or more hours subsequent to the injection of insulin, it has little, if any, beneficial effect on either incidence of rumplessness or toxicity of the insulin. No similarly beneficial results followed phosphopyruvic-acid supplementation (2 mg/egg); in fact, the toxicity in combination with insulin was very high and the incidence of rumplessness increased.

TABLE 8

Experiments concerning the effect of pyruvic acid on insulin-induced micromelia and beak defects in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ undiluted pyruvic acid

GROUP	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICROMELIA	ABNORMAL BEAK	ABNORMAL EYES
			%		%	%	%
1	Pyruvic acid — 117 hours insulin — 120 hours	405	71.9	74	75.7	29.7	5.4
2	Insulin and pyruvic acid — 120 hours	425	69.6	77	71.4	11.7	2.6
3	Insulin — 120 hours	225	20.0	168	33.3	4.8	0.6
4	Insulin — 120 hours pyruvic acid — 123 hours	396	77.3	52	75.0	17.3	0
5	Pyruvic acid — 120 hours	137	83.2	23	0	0	0

The effect of pyruvic-acid supplementation is very different at the time when insulin treatment leads to the occurrence of micromelia and beak defects (table 8). Pyruvic acid, given simultaneously with insulin-injection or within three hours prior or subsequent to it, greatly increases, at this stage of development, embryo mortality as well as incidence of micromelia and beak defects. All differences in these respects between group 3 (insulin alone) and the other three groups are statistically highly significant, except for that relating to the incidence of beak abnormalities in groups 2 and 3. The toxicity

TABLE 9

Experiments concerning the effect of lactic acid on insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ lactic acid (40%)

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS
			%		%
1	Insulin — 24 hours	336	42.0	181	24.3
2	Insulin and dl-lactic acid — 24 hours	334	20.4	235	12.8
3	Insulin and d-lactic acid — 24 hours	355	18.3	265	18.1
4	Insulin and dl-lactic acid — 24 hours	368	15.2	289	13.1

which was encountered in these experiments appears to be wholly due to the pyruvic acid. When pyruvic acid was injected by itself at 120 hours, mortality to the end of the second week amounted to 83.2% out of 137 treated embryos. No abnormalities of any kind were, however, found among the survivors. Phosphorpyruvic acid, given by itself, was harmless at this stage. In combination with insulin it increased embryo mortality without changing the incidence of abnormalities.

The results of experiments with lactic acid are shown in tables 9 and 10. When insulin treatment at 24 hours was

TABLE 10

Experiments concerning the effect of dl-lactic acid on insulin-induced micromelia and beak abnormalities in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ lactic acid (40%)

GROUP	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY FROM 96 HOURS TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICROMELIA	ABNORMAL BEAK	ABNORMAL EYES
			%		%	%	%
1	Lactic acid — 117 hours insulin — 120 hours	234	44.0	129	34.9	6.2	0
2	Insulin and lactic acid — 120 hours	221	59.7	78	41.0	12.8	1.3
3	Insulin — 120 hours	239	18.4	200	51.5	11.0	0.5
4	Insulin — 120 hours lactic acid — 123 hours	228	50.9	111	49.5	8.1	0.9
5	Lactic acid — 120 hours	148	54.7	67	0	0	0

supplemented by injection of racemic lactic acid, the incidence of rumplessness and embryo mortality were reduced to about one-half of that occurring after treatment with insulin alone. The differences are in both instances highly significant (between groups 1 and 2 χ^2 for embryo mortality was 36.451 with $P<.0001$, and for incidence of rumplessness $\chi^2 = 9.312$; $P<.01$). A repetition of the experiment with dl-lactic acid gave closely similar results (group 4), but with d-lactic acid (Pfanstiehl), the naturally occurring form, the results were less favorable than with the racemic acid.

The results of lactic-acid supplementation of insulin-treatment at 120 hours are shown in table 10. When racemic lactic acid was given three hours prior to insulin injection, the incidence of micromelia was reduced significantly ($\chi^2 = 8.708$, $P<.01$); there were also fewer beak abnormalities, but in this instance the difference was not statistically significant. Injection of lactic acid simultaneously with or three hours subsequent to insulin-treatment had no significant effects on incidence of either micromelia or beak abnormalities. It can be seen, on the other hand, that the combined treatment with lactic acid and insulin produced in all groups increased embryo mortality (in all instances $P<.0001$). This is accounted for by the high degree of toxicity which lactic acid alone has at this stage of development (group 5). As was true for pyruvic acid, lactic acid reduced the mortality from insulin treatment in early embryonic stages, but had a highly toxic effect in later ones.

Sodium acetate in a dose of 10 mg/egg was toxic, without producing abnormalities, but when injected together with insulin resulted in an exaggerated incidence of the teratologic effects of insulin as well as heightened embryo mortality. This was especially true at 24 hours, but to a lesser extent also at 96 hours.

Amino acids

We performed some experiments with l-tryptophane (1.2 mg/egg), dl-methionine hydrochloride (3 mg/egg), l-glutamic acid hydrochloride (12.5 and 25 mg/egg) and dl-alanine (7.2 mg/egg). When administered by themselves and in the given amounts, none of these amino acids, with the possible exception of l-glutamic acid hydrochloride, was teratogenic at either 24 or 96 hours of development. Except for l-glutamic acid hydrochloride, no striking toxicity was observed after injection of these compounds. At 24 hours, l-glutamic acid hydrochloride was somewhat toxic and pronouncedly so at 96 hours. Methionine, tryptophane and alanine in combination with insulin led to increased embryo mortality and among the survivors the incidence of rumplessness was higher than after insulin alone. At 96 hours these three amino acids probably produced no significant change in embryo mortality, nor in the teratogenic effects of insulin. A more interesting situation obtains in regard to supplementation with l-glutamic acid hydrochloride. Injected alone, this compound showed some toxicity for 24-hour embryos ($19.5 \pm 3.45\%$ mortality during first 6 days) and probably had a slight rumplessness-inducing effect ($6.5 \pm 2.46\%$). We made three tests at 24 hours in which the injection of two units insulin was supplemented by 12.5 mg/egg l-glutamic acid hydrochloride and two tests in which 25 mg/egg of this substance were given in addition to insulin. Since there was no apparent inconsistency between the results for the two different dosage levels, we are here reproducing only the totals for all 5 tests (table 11). It can be seen that l-glutamic acid hydrochloride supplementation reduced mortality as well as incidence of rumplessness. At 96 hours l-glutamic acid hydrochloride supplementation sharply increased embryo mortality, but did not change the teratogenic action of insulin.

Sodium succinate and 3-hydroxy-anthranilic acid

Some substances which are toxic and growth-retarding, but do not by themselves produce any teratological results, potentiate strikingly the incidence of insulin-induced micromelia and beak abnormalities when given together with in-

sulin at 96 or 120 hours of development. This is true, for instance, for adrenal cortex extract (Landauer, '47a). There are other substances which produce a similar potentiation of insulin action, but, when given alone and in the same amounts, neither affect viability nor interfere with normal development. Among compounds of the latter type are sodium succinate and 3-hydroxy-anthranilic acid. In experiments with 96-hour embryos 91.9% of all embryos survived the 17th day after the injection of 4 mg 3-hydroxy-anthranilic acid: among the 147 survivors the only abnormalities found were two cases of micromelia and one of cleft palate. After the injection of 8 mg

TABLE 11

Results of 4 tests with insulin alone and in combination with l-glutamic acid hydrochloride at 24 hours of development. Two units insulin all tests; l-glutamic acid hydrochloride 12.5 and 25 mg/egg, respectively, in different tests. White Leghorn eggs. Standard errors.

TREATMENT	FERTILE EGGS	MORTALITY DURING FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESSNESS
		%		%
Insulin	667	37.0 ± 1.91	391	24.3 ± 2.15
Insulin + l-glutamic acid hydrochloride	831	24.8 ± 1.50	600	13.3 ± 1.38
Difference		12.2 ± 2.43		11.0 ± 2.55

sodium succinate 88.8% of all embryos survived the 17th day and among the 143 survivors one rumpless chick was the only abnormality observed. When the same amounts of sodium succinate or 3-hydroxy-anthranilic acid were injected simultaneously with insulin, we obtained the results shown in table 12. For comparison we give the data for 4 tests with insulin alone, done during the same period. It can be seen that the incidence of all malformations was greatly exaggerated by the presence of either sodium succinate or 3-hydroxy-anthranilic acid. This potentiating effect was relatively the greater, the lower was the frequency with which a particular type of abnormality occurred under the influence of unsupplemented

TABLE 12

Data concerning the potentiating effect of sodium succinate and 3-hydroxy-anthranilic acid on the teratogenic action of 2 units insulin at 96 hours.

White Leghorn eggs

TREATMENT		SURVIVORS OF 17TH DAY	MICROMELIA	SYNDACTYLISM	BEAK ABNORMALITES
			%	%	%
Insulin + 8 mg sodium succinate 5/23/50		95	84.2 ± 3.75	18.9 ± 4.02	34.7 ± 5.17
Insulin + 4 mg 3-hydroxy-anthranilic acid 6/20/50		65	89.2 ± 3.86	36.9 ± 6.0	69.2 ± 5.75
Insulin (4 tests) 5/23 to 6/27 1950	Mean	425 [1]	60.0 ± 2.38	4.5 ± 1.01	26.1 ± 2.13
	Range	96–123	49.0–66.3	2.4–6.3	12.2–37.5

[1] Total of the 4 experiments.

insulin injection. For combined treatment with insulin and 3-hydroxy-anthranilic acid the degree of potentiation in terms of the mean incidence in the unsupplemented insulin control groups is demonstrated by the following figures.

	INCIDENCE AFTER INSULIN ALONE	POTENTIATION DUE TO 3-HYDROXY-ANTHRANILIC ACID
	%	%
Micromelia	60.0	48.7
Beak defects	26.1	165.1
Syndactylism	4.5	720.0

Syndactylism shows the most extreme effect. Syndactylism, not heretofore reported as an insulin-induced abnormality and to be discussed in more detail subsequently, was found in about 4.5% of Leghorn embryos which had been treated with two units of insulin at 96 hours. When, in addition to insulin, 8 mg of sodium succinate or 4 mg of 3-hydroxy-anthranilic acid were injected, the incidence of syndactylism rose to 18.9% and 36.9%, respectively.

Results with sodium succinate at 24 hours were very similar to those at 96 hours. Given by itself, it was non-toxic and non-teratogenic; added to insulin treatment, it greatly increased embryo mortality and incidence of rumplessness. The effect of 3-hydroxy-anthranilic acid at 24 hours was more complex. When this compound was injected by itself (4 mg/egg), it showed some toxicity (mortality during first 6 days $29.5 \pm 3.5\%$) and among the survivors of the 17th day we found 11.9% rumpless embryos. In a preliminary test with the same dosage the frequency of rumplessness had been 10% and for the combined data we have an incidence of $11.4 \pm 2.59\%$. It is evident that 3-hydroxy-anthranilic acid is by itself relatively potent in inducing rumplessness. Combined injection of insulin and 3-hydroxy-anthranilic acid resulted in very high mortality (84.4% during the first 6 days), but among the small number of survivors the incidence of rumplessness was not remarkably high (18.2%), possibly on account of differential mortality. On the other hand, this treatment led to the occurrence of some cases of micromelia, cleft palate and syndactylism, none of which were at this developmental stage produced by two units of insulin alone.

DISCUSSION

The major results of our experiments are as follows. A repetition and extension of earlier experiments with nicotinamide showed anew that, if proper dosage levels were used and the amount of administered insulin was not too great, supplementation with this vitamin gave complete protection against the usual teratogenic effects of insulin treatment at 96 or 120 hours of incubation. In spite of the fact, however, that supplementary nicotinamide prevented the occurrence of micromelia and beak defects[1] among the survivors to late stages, the post-operative mortality was not reduced. To a certain extent nicotinamide also protected genetically polydactylous embryos against the modifier-like effects that insulin has on the expression of this mutation. Lactic acid, when given three hours prior to insulin treatment, reduced the incidence of micromelia, but had little influence on the frequency of beak abnormalities. Lactic acid had no protective value when administered simultaneously with or subsequent to insulin. In all these situations the combined use of insulin and lactic acid was, however, highly toxic. In contradistinction to the effects of lactic acid, supplementation of insulin-treatment with citric acid (when given simultaneously) did not change the incidence of micromelia, but furnished considerable protection against the occurrence of beak defects. Results similar to those with citric acid were obtained with glucose-1-phosphate, oxalacetic acid and probably fructose-6-phosphate.

[1] These malformations of the beak have nothing in common with the post-mortem beak abnormalities reported by Blattner and Williamson ('50).

In accord with earlier observations we found that during the rumplessness-inducing period of insulin (24 hours of incubation) nicotinamide gave some protection to embryos, but that its effectiveness was much lower than at the later stage and that, in addition, it was definitely toxic at this earlier time. Supplementary pyruvic acid, on the other hand, sharply reduced the incidence of rumplessness and greatly lowered the toxicity which follows treatment with insulin alone. Among many substances tested, pyruvic acid was most effective in this respect. Results of a similar nature, but at a lower level of efficiency, followed supplementation with lactic and citric acid; l-glutamic acid hydrochloride gave a slight, but significant, amount of protection. From present and earlier evidence we may conclude that the sequence of protection against insulin-induced rumplessness, beginning with the most effective compound, is about as follows: pyruvic acid > lactic acid = citric acid > nicotinamide > α-ketoglutaric acid ≧ l-glutamic acid hydrochloride.

It has been shown in previous communications that the production of micromelia and beak defects can be achieved by treatment with eserine, sulfanilamide or insulin and that in all three instances supplementary nicotinamide protected embryos against the teratogenic action of the three compound-

(Landauer, '49; Zwilling and DeBell, '50). We could further demonstrate that the rumplessness-inducing action of sodium cacodylate and insulin can similarly and to a large extent be forestalled by added pyruvic acid. It seems justified to draw three conclusions from this evidence, viz., (1) that, as pointed out by Ancel ('45b, '50), certain chemical compounds have more or less specific teratogenic potentialities; (2) that insulin has several different potentialities depending on developmental stage; and (3) that, irrespective of the teratogenic agent employed, the same supplements were effective in forestalling the occurrence of a particular malformation or in reducing its incidence.

There is additional evidence in support of these conclusions. The two substances that in Ancel's ('45a, '50) experiments most regularly produced micromelia and beak defects, viz., eserine and sulfanilamide, were also the ones that led most frequently to the occurrence of syndactylism. We had not previously reported on the production of syndactylism by insulin, but our present observations show that during the stages when insulin treatment leads to the occurrence of micromelia and malformations of the beak, it is also responsible for the appearance of a certain incidence of syndactylism. Nicotinamide protects the embryo against this abnormality as well as against the other symptoms of insulin-treatment typical for the 96- or 120-hour stages. Syndactylism thus represents another malformation that can be produced by three different and unrelated chemical compounds and that in all instances can be forestalled by the same supplement. It is of particular interest to note that insulin-induced syndactylism always involves the 3rd and 4th toes, as does hereditary syndactylism. This is additional evidence for our earlier conclusion that the majority, perhaps all, of the insulin-induced abnormalities are of the nature of skeletal phenocopies. Hereditary syndactylism was believed to be inseparable from brachydactylism and feathering of the feet (Danforth, '19; Jaap, '39), but no instances of such association occurred in our material and Warren ('50) has recently

described a hereditary form of syndactylism, unaccompanied by these traits.

Among the beak defects produced by insulin one can distinguish 4 different types, viz., parrot beak (associated with a moderate shortening of the lower beak), short upper beak, cross-beak and cleft palate. The proportions in which these defects occur vary considerably from experiment to experiment, probably parallel with minor differences in the developmental stages obtaining at the time of treatment. All 4 types of beak defects are prevented by supplementary nicotinamide. We had illustrated a case of cleft palate and facial coloboma in an earlier publication (Landauer, '47b, fig. 4) but did not then describe it in detail. Ancel could produce cleft palate with trypaflavin, but with none of the other substances tested by him, in particular not with eserine and sulfanilamide, the two compounds leading to a high incidence of micromelia and parrot beak. This is, therefore, another instance in which insulin has the greater teratogenic latitude and in which it has been shown that nicotinamide-preventable abnormalities may be separately produced by unrelated chemical compounds. It cannot be doubted that these various substances, producing the same malformations and being made non-teratogenic by the same supplements, create their effects via identical or closely similar pathways.[2]

Throughout our observations it has become evident, however, and we shall have further occasion to discuss it, that, in spite of these similarities in the teratogenic action of groups of substances, there are many minor, but significant, dissimilarities in their effects. We shall here call attention to two examples concerning the position in time of the maximum susceptible period for certain compounds. It was shown earlier that the incidence of rumplessness after insulin treat-

[2] In mammalian embryos cleft palate has been produced experimentally in rats with trypan blue (Gillman, Gilbert and Gillman, '48), maternal riboflavin deficiency (Warkany, Nelson and Schaffenberger, '43), and maternal folic acid deficiency (Evans, Nelson and Asling, '51), in mice as a consequence of anoxia (Ingalls, Curley and Prindle, '50). Some of these techniques may in their biochemical effects be related to those described for chicken embryos.

ment increases from 0 hours (unincubated eggs) to a maximum early in the second day of incubation (Landauer and Bliss, '46). In contradistinction, sodium cacodylate has a significantly higher rumplessness-inducing effect at 0 hours than later. It is also of interest that following treatment with sodium cacodylate the protective value of nicotinamide seems to decrease during the same period (0–24 hours). A difference similar to that between sodium cacodylate and insulin, in regard to the production of rumplessness, exists between sulfanilamide and insulin with reference to the origin of micromelia. The incidence of micromelia after insulin-treatment rises to a maximum at approximately 120 hours of incubation (Landauer and Bliss, '46), whereas sulfanilamide-induced micromelia reaches its peak frequency at about 48 hours with a steep decline thereafter, the incidence following injection at 120 days being only about one-quarter that at 48 hours (Zwilling and DeBell, '50). In spite of these great differences in the stages of maximum effectiveness, nicotinamide completely forestalls the occurrence of micromelia in both instances. It is difficult to escape the conclusion that these time dissimilarities of chemical action are caused by effects on different, if closely related, links of the same metabolic chain, the links in question presumably having varying biochemical importance at one time or another, but damage to the chain being in either event repairable by identical supplements.

Of apparently quite another order are the events that, at different developmental stages, produce distinctive and dissimilar abnormalities, such as rumplessness or micromelia. Attention has already been called to the fact that, according to Ancel's observations, some chemicals have "specific" teratogenic effects. It does not reduce the importance of this finding that in those instances where well-defined stage-response differences exist, as with insulin, the stage "specificity" can to some extent be overcome by an increased dose of the teratogenic agent or by supplementation with substances that by themselves are non-teratogenic. For, even

with such substances as insulin, it is clear that the response differences of the embryo in different developmental stages are not merely the effect of an interference with identical physiological processes in altered morphological situations.

There is evidence of a varied nature for the conclusion that the origins of these stage and type-specific effects are separated by important changes in biochemical functions. To start with, it has been seen that striking differences exist in the protective efficiency of various supplements, such as pyruvic acid being best in forestalling rumplessness, irrespective of the chemical character of the teratogenic agent, and nicotinamide being the most successful protective substance against micromelia and beak defects, again without regard to the chemical constitution of the teratogenic compound. The nature of these differences will be further discussed below. Secondly, altered biochemical situations are evident from the toxicity relationships of various supplements. It has been shown, for instance, that pyruvic acid, while reducing insulin toxicity (as well as its teratogenic effects) during the rumplessness-inducing stages and being quite harmless when given alone, becomes extremely toxic (and loses its protective properties) probably prior to the establishment of circulation and certainly by the time when insulin and other chemicals lead to micromelia. Similar situations exist for lactic, citric and l-glutamic acid. Conversely, substances such as nicotinamide and glucose-1-phosphate, are definitely toxic during the early embryonic stages when they have little or no protective value as far as the origin of rumplessness goes, but are non-toxic later when they serve to reduce or prevent the production of micromelia and/or beak abnormalities. All these statements can, of course, be only made with assurance for the dosages used in our experiments. Still other evidence of a similar kind comes from the distinctive manner in which various substances potentiate the teratogenic insulin effects at one stage, but not at another. Thus adrenal cortex extract has no influence on the manner of occurrence of rumplessness after insulin, but forcefully potentiates incidence and ex-

pression of the abnormalities induced by insulin during the 4th or 5th day (Landauer, '47a); contrariwise, phosphopyruvic acid (2 mg/egg) and glutamine (1.8 mg/egg) greatly exaggerate the effects of insulin during the rumplessness-inducing period (unpublished data), but do not produce similar results when added to insulin during the micromelia-inducing stages. All these observations point to the existence of important physiological dissimilarities in the origin of abnormalities during the two developmental periods.

Even at one and the same stage one finds response differences to supplementation which any attempt at interpretation must take into account. Insulin treatment at 96 or 120 hours produces micromelia, beak defects and syndactylism, the incidence of the three types of defects decreasing in the given order. These variations in frequency of occurrence may be determined by developmental differences between the several primordia at the time of treatment or by dissimilarities in their quantitative dependence on the processes that are disturbed by insulin treatment. There are indications, however, that even in these situations qualitative variations in local functions may play an important role. This is suggested by our evidence for differential protection and differential potentiation following treatment with insulin and various supplements. Instances of differential protection (in experiments at 96 hours of incubation) include our observation that lactic acid supplementation reduced the incidence of insulin-induced micromelia, but not that of beak defects, or, that citric acid, glucose-1-phosphate and yet other substances protect against insulin-beak defects, but not against micromelia. Differential potentiation is well illustrated by the fact that supplementation of insulin treatment at 96 hours with 3-hydroxy-anthranilic acid produced relatively the greater potentiation the lower was the incidence of a particular abnormality after insulin alone (order of potentiation: syndactylism>beak defects>micromelia). Sodium succinate supplementation gave similar results. It would be difficult to interpret these observations in any fashion other than the occurrence of differential biochemical effects of insulin treatment on various organs and parts. It is quite possible that the primary action of insulin is the same in all instances, but that the results flowing from it vary according to the metabolic importance of the affected substance at the time of treatment and that the effectiveness or toxicity of supplements is in part determined by changing substrate requirements.

The extreme complexity of the teratogenic mechanisms that are responsible for our results is sufficiently clear. At the present stage of almost complete ignorance about the biochemical processes involved in morphogenesis and differentiation one cannot expect to define more than the most general outlines of the events that took place under the conditions of our experiments. It cannot be doubted, at any rate, that the principal, if not only, primary disturbance which, in our material, leads to abnormal development is an interference with embryonic carbohydrate metabolism. This is evident from the nature of the teratogenic substances, from the type of compounds which have preventative effects when used as supplements, and from the kinds of substances acting as potentiators of the teratogenic agents. Additional evidence from experiments with inhibitors of glycolysis will be presented below. This general conclusion is, of course, in good agreement with what is known about the importance of carbohydrate utilization in early development.

Our results also demonstrate clearly, however, that between our "early" (0 or 24 hours) and "late" (96 or 120 hours) stages of experimentation important differences exist in the manner in which the embryonic carbohydrate metabolism is interfered with by our procedures. Before proceeding with a discussion of these differences, it will be well to present the results of experiments with various inhibitors of anaerobic glycolysis. We tested the effect of sodium malonate, sodium fluoride, glyceraldehyde, phlorizin and monoiodoacetic acid on development of the chicken embryo at 0, 30 and 96 hours. The results are shown in table 13. This table indicates only the incidence of rumplessness at 0 and 30 hours and of

The stages during which rumplessness is most readily produced by teratogenic substances occur prior to the existence of circulation, and the action of these substances is presumably directly on cellular functions of the embryo itself. At the later period, however, when the embryo is likely to respond with micromelia and beak abnormalities, the circulatory system is well established. It is true that even at this stage the endocrine regulation of carbohydrate utilization has not yet come into being, the embryos in this respect still resembling the hypophysectomized and pancreatectomized adult mammal, but the yolk sac membrane has begun to function as "transitory liver." The investigations of Zwilling ('51b) have shown that one of the agents inducing micromelia and beak abnormalities, viz. insulin, has profound effects on the carbohydrate storage functions of the yolk sac membrane, thereby producing hypoglycemia and carbohydrate starvation of the embryo (Zwilling, '48, '51a b). Whether or not the disturbances of yolk sac membrane functions are the only primary consequence of insulin treatment at this stage, carbohydrate deficiency and malformations of the embryos being a consequence, remains to be determined. It seems at present more likely that the teratogenic agents responsible for micromelia and beak abnormalities have direct, as well as indirect, effects on the embryo since a similar syndrome of defects (and one also preventable by nicotinamide supplementation) follows sulfanilamide treatment in the absence of hypoglycemia (Zwilling and DeBell, '50).[4] Moreover, the same syndrome or similar ones can be produced by treatment with eserine sulfate or by deficiencies of the maternal diet in biotin, riboflavin and manganese, none of which conditions are known or likely to be accompanied by changes in the blood sugar level.

Taking into account the great stage-dependent differences in effect which supplementary substances, such as nicotinamide and pyruvic acid, produce in conjunction with insulin,

[4] It is interesting in this connection that according to Mendes Alves ('46) sulfanilamide and other sulfa compounds reduce the hypoglycemic effects of insulin and that this anti-insulin action is removed by nicotinamide.

and in view of th[illegible]rs of anaerobic glycolysis which are teratogenic [illegible]lopment become non-teratogenic later on, it must be concluded that during the transition from the 24-hour stage to that at 96 hours important changes have occurred in the normal metabolic activities of chicken embryos. It may be suspected that the altered responses are related to the increasing importance of aerobic respiration, as indicated *inter alia* by the sudden rise in cytochrome oxidase after the third day of development (Albaum and Worley, '42; Albaum, Novikoff and Ogur, '46). Establishment or increasing importance of the tricarboxylic acid cycle may be a factor.

In view of the high efficiency with which nicotinamide protects, between 96 and 120 hours of embryonic development, against the teratogenic effects of insulin, there can be little doubt that during this period insulin interferes with codehydrogenase activity.[5] This is, in any event, the only established function of nicotinamide (via the pyridine nucleotides). It seems likely that, as in the earlier rumplessness-producing stages, insulin temporarily reduced or abolished existing coenzyme supplies, possibly by competing for the apoenzyme, but that at this later stage with its different respiratory activities nicotinamide (rather than pyruvic acid) is needed as a supplement in order to insure the availability of adequate amounts of coenzyme. Most of our observations with various supplements, whether beneficial or harmful, can be reconciled with this view. In particular does it become understandable that nicotinamide, in excessive amounts (equal amounts being more excessive in early stages), is likely to be toxic and teratogenic by acting as an inhibitor of its own coenzyme.

Certain pathways which might be suspected to play a role in the origin of our malformations are ruled out by available evidence. Inhibition of succinic dehydrogenase activity seems excluded since several inhibitors of this system (sodium

[5] Measurements are in progress.

malonate, triphenyl tetrazolium chloride and oxalacetic acid) have no teratogenic effects on 96-hour chicken embryos, even when given in toxic amounts.

Since eserine is one of the substances producing micromelia and beak defects, it might be asked if the origin of these abnormalities is related to anticholinergic action. This is very unlikely for two reasons. The anticholinergic consequences of eserine presumably occur after much smaller amounts of the drug than Ancel and ourselves found effective for the production of abnormalities. Furthermore, unpublished experiments with diisopropylfluorophosphate (0.2 cm^3/egg of 0.1% solution) at 96 hours gave no indication that this anticholinergic substance is teratogenic. It has been shown, on the other hand, that eserine sulfate, in such concentrations as are needed for the production of micromelia, leads to interference with processes of dehydrogenation (Brooks, Rensmeier and Gerard, '49), and it is in better accord with all our other evidence to assume that a mechanism of this kind is responsible for the micromelia-inducing action of eserine.

It is certain that the ultimate results of those biochemical changes which are impressed upon embryos by teratogenic substances may be modified in various ways by conditions existing in the embryos themselves, but we have little specific information about the manner in which these modifications are produced. One such modifying agency which has been noted again and again in our work is related to the age of the pullets from which eggs had been obtained. It finds expression in a gradual decline in response of embryos to insulin from early spring to mid-summer. This has been found true with regard to the incidence of rumplessness as well as of micromelia and beak defects. It seems likely that this change of response is produced by a regular trend of some kind in the composition of eggs, such as has been recorded by Csonka ('50) for nitrogen, methionine and cystine content.

Another source of variation in response to teratogenic substances, relating more to manner and degree of expression

than to incidence, is probably to be found in the exact developmental (and therefore presumably biochemical) condition of a particular organ primordium at the moment at which a teratogenic substance takes effect. Such relatively minor differences in developmental age presumably decide in which of several alternative morphological patterns rumplessness arises after insulin treatment (Moseley, '47) or in which of several possible directions beak development deviates from the norm. The lesser effectiveness of nicotinamide in preventing insulin-induced changes of expression of polydactylism as compared with its protective value against micromelia may be related to the nearness of irreversible determination of the toe primordia at the time of treatment. However, at this same time, i.e. 96 hours of development, it is still possible to produce in normal-toed stock a differential rise in the incidence of insulin-induced syndactylism by giving 3-hydroxy-anthranilic acid in addition to insulin. The developmental origin of syndactylism presumably occurs very soon after that of polydactylism. It seems, therefore, that for changes in the pattern of toe development the time of maximum sensitivity falls near the moment of irreversible differentiation.

A different situation obtains in regard to growing bones. Insulin treatment produces micromelia and, if less frequently, beak defects of chicken embryos as late as 168 hours of development (Landauer, '47) and probably still later. The long duration of susceptibility of the growing long bones to insulin is presumably accounted for by the special importance of glycogen storage in cartilage (for review see Follis and Berthrong, '49) and the existence of a phosphorylating system associating glycogenolysis with calcification (Gutman and Gutman, '41; Gutman, Warrick and Gutman, '42). Reduced glycogen stores and/or impaired glycogenolytic activity probably slow down calcification and, indirectly, many other processes of growing bones. Interference of this kind can be expected from the time calcification begins (or perhaps even earlier) to the end of cartilage replacement by bone. Hence

the protracted effectiveness of insulin in calling forth micromelia and allied defects. It remains to be explained, however, what causes the differential response of various skeletal parts, e.g. why, irrespective of the time of treatment (and also in all the mutant forms), the long bones of the leg are more affected by abnormal conditions than are those of the wing.

Many other details of our observations must remain unexplained for the time being. It is not clear, for instance, why l-glutamic acid hydrochloride affords a significant, if low, degree of protection against rumplessness after insulin-treatment, but tends to produce a certain incidence of rumplessness, when given by itself.

It is a common experience that substances of very different chemical constitution have similar pharmacological effects, but only in exceptional cases has their common pathway been discovered. This has often led students to the premature conclusion that common pathways do not exist. In the analysis of biological problems such situations have frequently been disposed of with the inference that particular responses are of an "unspecific" nature, a concept to which many shades of meaning became attached.

The idea that an "unspecific" retardation or arrest of development is the cause of malformations has a long history in which appear the names of Harvey, Haller, C. F. Wolf, Etienne and Geoffroy Saint-Hilaire, and Dareste. Its best known modern proponent, C. R. Stockard ('21) put it in the following words: "First, all types of monsters, double as well as single, may be caused by one and the same treatment; second, any one type of monster . . . may be produced by a great number of different experimental treatments; third, all effective treatments tend primarily to lower the rate of development, and, fourth, the type of monster induced depends upon the particular developmental moment or moments during which the developmental rate was reduced."

The work of Ancel and our own experiments have shown conclusively that treatment of chicken embryos at one and the same developmental stage will lead to different results

depending on the particular chemical agent to which the embryo was exposed, e.g. sodium cacodylate will give rise to rumplessness in 24-hour embryos, but not to micromelia at 96 hours, eserine sulfate or sulfanilamide will induce micromelia at 96 hours, but not rumplessness at 24 hours. It is a fact that one and the same type of abnormality frequently can be produced by several, and often chemically unrelated, substances, but wherever tests with supplementation have been applied, it could be shown that specific derangements of metabolism caused particular defects. This, of course, does not preclude the possibility that one and the same malformation may result from more than one type of biochemical interference. Nor is it justifiable to point, in support of Stockard's generalization, to our observation that one and the same compound, e.g. insulin, may produce several types of malformations depending on the developmental stage of treatment. For, our experiments with metabolic supplements make it clear that this versatility of insulin is due to a multiplicity of reactions rather than a lack of specificity.

If Stockard's third principle was meant to imply that *any* lowering of developmental rate at a susceptible period will *ipso facto* be responsible for the subsequent occurrence of malformations, it is demonstrably untrue as is shown by many cooling experiments with developing chicken embryos. But even where, following treatment with teratogenic substances, the emergence of morphological defects is associated with growth retardation, a causal relationship should not be assumed without proof. In the case of sulfanilamide, for instance, Zwilling and DeBell ('50) have shown that growth retardation and micromelia are independent and separable effects.

It is true that many of the teratogenic substances used by us are known to have inhibitory effects on mitosis. This has been shown, e.g., for insulin (Bullough, '49; Bullough and Eisa, '50), sulfanilamide (Fisher, Henry and Low, '44), arsenic compounds (King and Ludford, '50) and monoiodoace-

tic acid. Not only was it possible, however, to separate, in the case of sulfanilamide, growth retardation and teratogenesis, but many of our malformed embryos and chicks had normal body weight. Since the publication of Stockard's paper the origin of many abnormalities has been explained on the basis of interference with mitotic activity and embryo growth (e.g. Bodenstein and Abdel-Malek, '49), but in many of these instances closer scrutiny may yet trace the causal sequence to metabolic upsets within particular primordia.

Finally, the 4th of Stockard's principles can only be accepted with reservations. We could demonstrate, for instance, that the latitude of susceptible periods, in regard to a certain defect, varies with the dose of the particular teratogenic substance or that for different teratogenic agents the maximum period of susceptibility differs with respect to one and the same abnormality.

All our observations point to the conclusion that, as development proceeds, there are shifting substrate requirements and changing enzyme activities in different parts of the embryo and that the resulting internal competition is responsible for the occurrence of critical periods and their attendant dangers.

The functioning of particular enzyme systems and the availability of and needs for particular substrates presumably are inextricably interwoven during development and dependent on each other. But the immediate cause of the failure of certain organ primordia to develop normally after treatment with teratogenic agents must be looked for in a lack or insufficient supply of necessary substrate and it may be concluded from our observations that selective starvation plays an important role in the origin of malformations. The patter of abnormal development varies with the stage attained at the time of treatment, but differs considerably from that observed on explanted blastoderms (Spratt, '50). The selective susceptibility of skeleton-forming material is one of the outstanding features of our observations.

SUMMARY

Our observations can be summarized as follows.

With proper dosage and timing supplementary nicotinamide protected against the abnormalities (micromelia and beak defects) produced by insulin-injection at 96 or 120 hours of development.

Nicotinamide decreased to some extent the modifying action of insulin on the expression of hereditary polydactylism.

The incidence of rumplessness after insulin at 24 hours was only slightly reduced by supplementary nicotinamide.

At 24 hours, but not at 96 or 120 hours, embryo mortality after insulin-treatment was much increased by supplementary nicotinamide. At the earlier developmental stage nicotinamide had considerable toxicity when given alone and was responsible for the occurrence of beak abnormalities.

The incidence of beak defects after insulin at 96 hours could be selectively reduced by supplementary glucose-1-phosphate, citric acid, oxalacetic acid and, probably, fructose-6-phosphate. There was no similar lowering in abnormalities of the extremities (micromelia, syndactylism).

Pyruvic acid, when given as a supplement to insulin-treatment at 24 hours, greatly reduced the incidence of rumplessness and lowered embryo mortality. The same was not true for phosphopyruvic acid. Results similar to those with pyruvic-acid supplementation, if less spectacular, followed supplementation with lactic and citric acid. Isocitric acid was highly toxic and increased the incidence of rumplessness.

In later developmental stages pyruvic acid was very toxic and in combination with insulin potentiated the teratogenic effects of the latter. Lactic acid also became toxic at later stages, but, when given three hours prior to insulin, it still had some protective value.

The l-glutamic acid hydrochloride, when injected by itself at 24 hours, had a low rumplessness-inducing effect, but it reduced the incidence of rumplessness after insulin treatment when used as a supplement.

Sodium succinate and 3-hydroxy-anthranilic acid potentiated the effect of insulin at 96 hours. This potentiation was the greater the lower was the incidence of a particular abnormality after insulin alone. Given by themselves both compounds were harmless.

Malformations produced by insulin-treatment at 96 or 120 hours, and not heretofore reported, are syndactylism and cleft palate, the latter associated with coloboma of the face.

Tests with inhibitors of anaerobic glycolysis (sodium malonate, sodium fluoride, glyceraldehyde, phlorizin, monoiodoacetic acid) showed that phlorizin and especially monoiodoacetic acid, injected at 24 hours of incubation, produced a considerable incidence of rumplessness. None of these inhibitors were teratogenic at 96 hours.

Our results demonstrate that certain substances provide protection against those teratogenic compounds which in 24-hour embryos tend to induce rumplessness, that other substances forestall, completely or in part, the production by chemical interference at the 96- or 120-hour stage of micromelia, syndactylism and beak defects, that some supplements furnish differential protection (e.g. against beak defects, but not against micromelia), and that other supplements produce differential potentiation of the insulin effects (e.g. raising the incidence of syndactylism much more than that of beak abnormalities). It follows that the metabolic situations upon which act the teratogenic agents as well as the substances used as supplements, vary not only between different developmental stages, but also between different organ primordia of one and the same stage.

It seems likely that the teratogenic effects of insulin are produced by rendering coenzyme unavailable to the embryo. During the early and predominantly anaerobic stages of development pyruvic acid, by aiding reoxidation of the pyridine nucleotides, presumably serves to prevent insulin damage. During later stages, and on account of the changed respiratory conditions of the embryo, nicotinamide supplementation is required to offset the blocking effect of insulin on the co-

enzymes. Chemicals which resemble insulin in their teratogenic effects probably do so by interference with the same or closely related metabolic steps. Supplements to insulin treatment seem to be beneficial or noxious according to whether they aid or hinder in relieving the insulin determined scarcity of coenzyme.

The long duration of susceptibility to insulin treatment of the long bones and other skeletal parts can be explained on the basis of the special significance of glycogenolysis in bone calcification.

The principles of "unspecific" retardation of embryo growth, as enunciated by Stockard and others, cannot explain the origin of the teratogenic processes here described.

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EFFERVESCENT BEVERAGE POWDERS

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This invention relates to the art of manufacturing an effervescent beverage powder that is reconstitutable to a beverage of the cola or fruit-flavored type which simulates the soda-like taste sensation or carbonation "bite" of bottled beverages.

Heretofore, the art has long sought the provision of a beverage powder such as would reconstitute into a saturated solution of carbon dioxide. In the main, prior art workers have concerned themselves with the creation of an effervescent couple through the reaction of a carbonate and an acid. Although this mechanism has been available for many years, no commercially sold beverage powder has been provided which on drinking approximates the carbonation sensation of beverages of the cola type vended in air-tight glass or metal containers produced by the creation of a saturated solution containing several volumes of carbon dioxide. At best, beverages formed from these dry powders are characterized as rapidly "fizzing" products which, upon addition to water undergo rapid reaction of the effervescent acid-carbonate couple with an almost immediate loss of any available carbon dioxide. In fact, upon the passage of as short a period of time as 2 to 3 minutes a substantially saline beverage is experienced with little or no discernible sustained carbonation.

It is an object of the present invention, therefore, to provide a beverage mix which substantially simulates the beverage character of a supersaturated solution of carbon dioxide which is commonly sold as a fruit flavored or cola beverage.

A more particular object of the present invention is to provide a beverage powder in a form such as can be readily reconstituted either with natural or artificial sweeteners and sustains the carbonation effect stemming from the reaction of acidic components and carbon dioxide-containing salts without encountering a rapid "fizzing" and degassing of the solution thus produced.

The present invention is founded upon the discovery that when a relatively instantly soluble bicarbonate salt and a relatively instantly soluble form of a food grade acid are put into solution rapidly (say in a period of less than 5 seconds) a solution of the reagents thus produced creates a metastable solution of carbon dioxide and simulates the beverage quality of a bottled or canned carbonated drink of the cola or fruit-flavored type. In accordance with the present invention a relatively soluble form of a bicarbonate salt is finely divided to a particle size which is capable of going rapidly into solution in a period of preferably less than 3 seconds; correspondingly, an acid powder such as citric, malic, fumaric, adipic and the like, is suitably sized through subdivision to a finely particulate state so that it too goes into solution rapidly and essentially in combination with the liquid bodies resulting from solution of the bicarbonate results in an immediate reaction between the solution products. While the present invention is not to be understood as restricted to any particular theory in discerning the scope thereof defined in the accompanying claims, it is believed the rapid and instantaneous reaction between the individual bodies of bicarbonate solution and acid solution resulting from distributing the powders thereof into water gives rise to a metastable state such as results from addition of liquid sodium bicarbonate solution and a liquid acid solution.

It is a particular feature of the present invention that both the acid component and the bicarbonate component in powder form should be at an optimum particle size such that substantially no trace quantities of over-sized food acid powders or bicarbonate powders are left suspended in the reconstituted beverage. In this way the fizzing phenomenon manifest in effervescent beverage powders of the prior art is reduced and solution efficiency is increased many-fold. Thus, in the case of sodium bicarbonate it will preferably be provided in a form that has a relatively narrow particle size distribution while at the same time being finely divided so as to be available for rapid and substantially instantaneous solution in the reconstituting liquid. Dissolution time should be under 5 seconds and preferably 2 to 3 seconds. Likewise the food acid such as citric acid and various of the other edible grade food acids will be finely divided and have a narrow particle size range such that they too will be substantially uniformly dispersed in the reconstituting aqueous medium and no over-sized acid bodies will be left available after 5 seconds in the aqueous medium. Essentially, the weight percent of bicarbonate to citric acid powder depends upon the intended degree of carbon dioxide saturation intended for the beverage solution. For fruit-flavored beverages a lower degree of saturation will be called for than in the case of a cola type which is more usually in the supersaturated or highly saturated category. Qualitatively, there should be an over-abundance of the uniformly sized acid powder. An over-abundance of fruit acid should be used so as to impart the required sourness to complement the fruit or other flavor desired for the beverage produced upon reconstitution of the powder. The amount of carbonation obtainable will depend upon the bicarbonate element of the reagents. Further, the level of bicarbonate component of the beverage mix should, in accordance with the present invention, be substantially below that point where solubility of the quantity of bicarbonate powder by weight of the reconstituted liquid will endanger the instantaneous creation of bicarbonate solution. In other words, an excess of bicarbonate powder should not be left suspended after the beverage mix has been reconstituted otherwise a nucleating and degassing effect will ensue as the effervescent couple continues to react. The level of acid required will be predicated upon the degree of sourness required for the intended flavor and, as indicated previously, in any event will be in excess of that amount required to provide a complete and sufficient reaction in furnishing the carbon dioxide solution; thus, the acid should not and will not be completely neutralized in the course of creating the carbon dioxide solution.

The invention in its various embodiments will now be more fully described. One preferred embodiment of the invention involves grinding and sifting sodium, potassium or other water-soluble form of bicarbonate to a finely divided uniform state having an average particle size of about 400 mesh. The particle size must be small but at the same time be above that particle size where the particles themselves will clump or float upon attempted reconstitution in water. In general, the average particle size of these finely divided and sifted sodium bicarbonate powders will range from about 35 to 55 microns.

Similarly, the acid component of the effervescent couple will be finely divided to a narrow particle size distribution range and have a particle size substantially below that level whereat any insoluble bodies of acid powder will exist and at the same time be above that level where ... as "floaters" or finely suspended food powders will ... manifest such as will be available for reaction with ...

over-sized particles of bicarbonate. For most edible food grade acids such as citric, adipic, tartaric and the like, the particle size range will be such that 95% of the acid powder passes a number 50 mesh screen and will not be small enough to result in clumping. The particles should have an average mean diameter not greater than about 300 microns. In the case of the acid powders, they are less likely to float and clump and, hence, the smaller mesh of particle size is not as critical as with the bicarbonate powder. In any event, the acid powder should be subdivided to a particulate state whereat substantially all of the free bodies thereof will enter into solution for reaction in a period of less than 5 seconds and preferably in 1 to 3 seconds.

As an alternative form of the invention, the sodium bicarbonate may be dissolved in water and the solution subdivided to a finely particulate state and dried at below 100° F. This will result in a free-flowing powderous uniform finely divided form of bicarbonate powder. Thus, a water solution of sodium or potassium bicarbonate solution may be spray dried under conditions which yield a particle size distribution within the aforestated particle size range. In still another embodiment of the invention, the finely divided bicarbonate can be co-dried with a bulking agent of the water soluble type such as any one of the variety of mono-, di-, and polysaccharides typified by sucrose and dextrose. In this embodiment of the invention the bulking agent will be placed in solution (e.g., sucrose), the sodium bicarbonate powder will be dispersed therein and the solution will then be spray dried. Alternatively, a solution of sodium bicarbonate may be co-dried with a sugar solution (such as sucrose) to the aforestated particle size wherein the sodium bicarbonate powder will be rendered available for substantially rapid and instantaneous solution upon reconstitution with the acid component of the beverage mix.

The two dry mix components can be intermixed with sucrose or any other flavoring agent such as dextrose, lactose, corn syrup solids and the like or, alternatively, can be combined with an artificial sweetener such as saccharin, sodium cyclamates and saccaharin in combination with sodium cyclamates.

The powder should be packaged in a substantially fluffy condition such that the components are maintained as substantially discrete one from the other as possible, while avoiding any interaction such as may result through the presence of moisture during and after packaging. Accordingly, it is important that the dry mixes be packaged in a hermetic container such as substantially maintains whatever moisture equilibrium exists during initial packaging of the powderous material. In packaging the components of the present invention, care should be taken to assure that the discrete and finely divided state of the acid and bicarbonate components is maintained with a minimum of clumping or compaction so that upon opening the envelope or other container therefor they will be rapidly and instantly soluble with a minimum of nucleation and the concomitant fizzing and degassing effects which could ensue.

In accordance with the present invention, it is possible to obtain carbon dioxide efficiencies of about 85% of theoretical through reaction of the bicarbonate with the acid. This represents a markedly improved reaction efficiency in comparison to the beverage powders and pills now marketed for beverage carbonation purposes. Upon reconstitution the mix rapidly goes into solution upon simple spoon stirring with one stir. The mix itself may be added to warm or cold water (preferably cold) to maintain maximum carbon dioxide retention. The water may have a sweetening agent such as sucrose separately added to it by the consumer or sucrose may be present in the mix and added with the acid and bicarbonate component thereof. Accordingly, it is preferred in accordance with the present invention, that the sugar be sold as a component of the mix to assure the desired carbonation result is achieved. On the other hand, the housewife or other consumer can be instructed to place the sugar, sucrose or other sweetening agent into solution prior to addition of the mix thereto. Where the sweetening agent (e.g., sucrose) is placed in intimate admixture with the bicarbonate and acid components, the sugar, sucrose or other sweetening agent should be finely divided to a particulate state approximating that of the bicarbonate powder itself so as to assure that there is virtually no nucleating agent present when the mix is reconstituted.

Upon reconstitution the beverage will effervesce for just an instant (say in the neighborhood of 1 to 2 seconds) during which time bubbles will ascend to give somewhat of a sparkling and visible effect of carbonation like that of a bottled carbonated beverage. However, unlike the fizz pills and powders of the prior art, this ebullition will only last for an instant and only very few bubbles will be apparent. Thereafter visible carbonation will cease completely and only several small bubble clusters will remain on the sides of the containing vessel. The beverage solution can then stand for a long period of time (10 to 30 minutes) without any ebullition of CO_2. Upon consumption of the beverage the consumer will experience a carbonation "bite" like that of bottled carbonated beverages due to release of gaseous CO_2 in the mouth. Indeed, it is at this point that the effervescent effect will be experienced and this will occur rapidly to furnish the required refreshment and novel sensation to complement the flavoring or other constituents of the beverage. As contrasted from the beverage or fizz pills employing an effervescent couple, the reconstituted mix will stand for extended periods of time without losing its inherent carbonation effect. The reconstituted beverage can stand for over an hour at room temperature without losing its carbonation properties, the carbon dioxide thereof being lost very slowly.

The invention will now be more fully described by reference to the accompanying detailed operative example.

EXAMPLE 1

A one volume beverage concentrate

Constituents:	Grams
Precipitated sodium bicarbonate (45 micron average mean diameter)	900
Anhydrous powdered citric acid (297 micron average mean diameter)	1390
Sodium cyclamate-saccharin (3:1 ratio)	150
Flavor	9
Color	1

The citric acid and sodium bicarbonate are mixed and then tumbled within a smooth drum 10 inches in diameter and 15 inches long, turning about a horizontal axis parallel to the longitudinal axis of the drum. The speed of rotation is 100 r.p.m. The tumbling is carried out for about 5 minutes. Next the cyclamate-saccharin (3 parts cyclamate to 1 part saccharin), flavor, and color ingredients are added to the drum and tumbling continued for 1 to 2 hours at a humidity of 20 to 40% and a temperature of about 75° F.

In this manner a thoroughly blended and homogenous mixture of the finely powdered ingredients is obtained which can be packaged in small aluminum foil envelopes suitable for containing enough dry mix to form a 7 ounce beverage drink. Each envelope should contain about 2.7 grams of the dry beverage concentrate in order to form a 7 ounce (200 cc. of water) carbonated beverage containing about one volume CO_2. One swish of the stirring spoon is enough to dissolve the beverage in 1 to 3 seconds to give a metastable one volume water solution of CO_2. The carbonated drink exhibits no visible effervescence or carbonation after a slight gaseous release of CO_2 which bubbles off on initial contact of the dry powder and water. The beverage solution is stable

on standing for 10 to 30 minutes and only gradually loses soluble CO_2 to the atmosphere. The beverage has a distinct carbonation "bite" when placed in the mouth after 15 to 30 minutes.

While this invention has been described with particular reference to the preferred alkali metal bicarbonates such as sodium and potassium bicarbonate as the carbonating agents to be used in practicing the invention it is understood that other forms of carbonating agents may be used such as sodium or potassium carbonate.

It will also be apparent to those skilled-in-the-art that although this invention has been described in terms of a specific example, that various modifications may be made thereto which will fall within the scope of the appended claims.

What is claimed is:

1. A rapidly dissolving, dry effervescent concentrate adapted to form a carbonated beverage with water, comprising a plurality of ingredients including an acid component and a carbonating component, said acid component including particles of an edible food acid having an average mean diameter not greater than about 300 microns and said carbonating component consisting essentially of particles of an alkali metal bicarbonate having an average mean diameter not greater than about 55 microns, all of said ingredients being soluble in water with agitation in less than 5 seconds.

2. A concentrate as claimed in claim 1, in which said food acid is selected from the group consisting of citric acid, adipic acid and tartaric acid.

3. A rapidly dissolving, dry effervescent concentrate adapted to form a carbonated beverage with water, comprising a plurality of ingredients including an acid component and a carbonating component, said acid component including particles of an edible food acid having an average mean diameter not greater than about 300 microns, and said carbonating component consisting essentially of particles of an alkali metal bicarbonate having an average mean diameter of about 35 to 55 microns, said acid component being present in an amount in excess of that required to completely react with said carbonating component, all of said ingredients being soluble in water with agitation in about 1 to 3 seconds.

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ACIDULANTS USED IN FOODS

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Tartaric acid extracted from grape must and citric acid extracted from citrus fruits have been used as acidulants in foods since long ago. They were practically the only acidulants used by the food industry for a long period of time. Now, with the increased consumption of acidified foods and as a consequence of the presence of more economical acidifying agents, an increased effort by industry to supply the consumer market of these acids with other acids, organic or inorganic, non-toxic in the quantities habitually used by man, to be used in foods has been observed. At that time two alternatives were available: 1) to extract or synthesize acids commonly present in natural foods; 2) to try to obtain other acidulants which were not usually present in comestible fruits and which were non-toxic elsewhere. The truth is that many existing organic acids, abundant in fruits and other comestible parts of vegetables, are toxic to animals causing pronounced nocuous effects, as is the case with oxalic acid.

An acid found in abundance in fruits of the Pirus class is maleic acid, very carefully studied in relation to its industrialization via synthesization when its effects were more or less known. Today, aside from the small quantities of natural L maleic acid at our disposition, we also have great quantities of synthesized DL maleic acid. Glycollic acid, contained in many fruits, was proposed for use as an acidulant after the second world war due to the facility of its preparation by synthesization from acetic acid.

Lactic acid, appearing in many fruits and animal organs, found widespread use as an acidulant in foods. The diffusion of its use is due to its economical manufacture by the fermentation of sucrose.

In the economic field much progress was made in the production of citric acid by the fermentation of sugar so that the consumption of this acid has been greatly increased.

Lastly, among the organic acids proposed and well accepted, gluconic acid stands out, commercially furnished in the form of a watery, syrupy solution. This acid is also found in many fruits, and is easily prepared by the oxidative fermentation of glucose. The acid itself, as well as its salts (of Ca, Fe, Cu), is extensively used in modern therapeutics with mineral salts.

The only inorganic acid proposed as an acidifying agent is phosphoric acid. This, as well as the other acids mentioned, has an agreeable taste and is not toxic in quantities habitually used, and is further recommended because it may be produced in a very pure form.

The first question raised at any proposal for a new acidulant to be used in foods is its degree of toxicity, and later, what other properties seem favorable to its use in alimentation, what is its grade of purity, flavor, aroma, etc.

The data on toxicity found in scientific literature is gathered in our present work. There are acids which have been very carefully studied in various laboratory animals in relation to their effects, acute or chronic toxicity, lethal doses, etc. Others however, in spite of much recognition, have not been approached from those aspects.

We will now examine each acid separately.

CITRIC ACID

There are works in scientific literature which sometimes argue against the indiscriminatory use of this acid.

Zipkin (1947) of NIH in studying the citric acid content in human saliva revealed that in each 100cm of saliva in the adult male there is from 0.6 to 2.0mg of citric acid. Zipkin suggests the existence of a relation between salivary citric acid and erosion of enamel and cavities. The decalcification of the tooth enamel by the citric ion is suggested by the observation that this ion with that of calcium forms a soluble ionized compound in the investigations of Shear, Kramer, and Resnikoff (1929). And Clure and Ruzicka (1946) observed that the citrate, in a practically neutral drink, had a pronounced destructive effect on the dental enamel "in vivo".

In opposition to the findings of Stafne and Lovestedt (1947), Newman (1948) verified that there was no evidence observed relating to the destruction of teeth which could be attributed to the effects of citric acids of citrus fruits. Experiments were conducted on Puerto Ricans who consumed an average of 4.24 oranges or grapefruits per day for 5 to 6 months a year.

Leschke (1932) states that citric acid has a hindering effect on blood circulation which closely ressembled that of oxalic acid, occasionally leading to grave intoxications, even death. It is particularly grave in children in whom circulatory deficiencies and unconsciousness could occur after the ingestion of even 1 or 2 lemons. The author mentions the case of a young woman who ingested 25g of citric acid in one time for a laxative effect; she began to vomit continuously and almost died.

As to the destiny of the ingested citric acid, Sherman, Mendel, and Smith (1936) conducted studies on laboratory animals, observing the possibilities of destroying large doses of citric acid in dogs. In the oral administration of 0.5 to 2.0g/kg of acid approximately 0.7% is not oxidized, appearing in the urine. The ingestion of the acid did not affect either the pH or the total nitrogen collected in the urine in 24 hours. Kuiper and Mattill (1933), in studies on men, concluded that when from 2 to 20mg of citric acid is ingested it is rapidly oxidized in the organism, only 1.5 to 2.5% remaining which is excreted in the urine.

The toxic effect of citric acids and its salts was recently determined by Gruber Jr. and Halbeisen (1948). They determined that the symptoms provoked by the administration of large doses of citric acid and citrates ressembled those of a calcium ion deficiency, which are an increase of general activity, the appearance of hyperemia, peripheral vasal dilation, salivation, clonic and tonic convulsions, cyanosis, and at times, death. The median lethal dose in millimoles/kg was determined in different animals. The results are shown in the following table:

	D.L. :millimoles/kg				
	Rabbits intraven.	Mice intraven.	Rats intraperit.	Rats intraperit.	Rabbits intraperit
Citric Acid	1.72	0.22	5.0	4.6	0.331
Sodium Citrate	1.76	0.23	7.6	6.3	
Bisodium Citrate	1.77	0.30	7.5	7.3	
Trisodium Citrate	1.74	0.60	5.5	6.0	

If we take the results of the D.L. for the rabbit administered intravenously and transmute them for a man of medium weight (70 kg) we will obtain D.L. = 7.1g/70 kg.

The chronic toxicity after a prolonged administration of citric acid was determined by Krop and Gold (1945), revealing that after receiving a dose of 1.38g/kg of acid for 103 to 108 doses (3 dogs) the animals did not present any symptoms of intoxication, even upon a histological examination. Such results, calculated for a man of medium weight of 70 kilos, would give us almost 63.6g daily as a non toxic dosage.

Through the work of Kuiper and Mattill already reported we know that an ingested dose of 2 to 20g of citric acid is quickly metabolized in the organism and that only 1.5 to 2.5% is excreted in the urine. Weiss, Downs, and Corson (1923) fixed the tolerable dose of maleic and citric acid by prolonged administration at 2.5g per kilo in rabbits, and at around 2g per kilo for tartaric acid. These data calculated for a man of 70kg gives us the daily doses which may be ingested for a prolonged time without damage to health:

Maleic and citric acid -- 53g/70kg
Tartaric acid -- 43g/70kg

DISCUSSION

The objective of the present work was to bring to light scientific truth concerning the toxicity of the mentioned acids, so that they may or may not be admitted as acidulants in food and drink, inter nos.

The Law 15.642 of 9/2/46 which approves the Regulation of the Supervision of Public Alimentation barely admits the use of citric, tartaric, and lactic acids as acidulants. In view of what has been exposed, it is easy to verify that some of the other acids could be included more readily than tartaric acid.

In the discussion presented and in accordance with the data obtained in literature, data resulting from experimental findings in laboratory animals, whether concerning acute or chronic toxicity, we may conclude the following:

Daily doses tolerable by prolonged administration in an adult of 70kg without damage to health:

1. Citric acid 53g
2. Maleic acid between 43 and 53g

3.	Lactic acid	median 43g
4.	Tartaric acid	43g
5.	Fumaric acid	approx. 43g
6.	Gluconic acid	17g
7.	Glycollic acid	18g
8.	Phosphoric acid	11g

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AGENTES ACIDULANTES UTILIZADOS EM ALIMENTOS

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Desde remotos tempos, têm sido utilizados o ácido tartárico extraído do mosto da uva e o ácido cítrico extraído de frutas do gênero *Citrus* como agentes acidulantes em alimentos. Durante longo tempo, empregaram-se, na indústria alimentícia, quase que exclusivamente êstes ácidos. Todavia, com o crescente aumento de consumo de alimentos acidificados e em conseqüência da procura de agentes acidificantes mais econômicos, observou-se um acentuado esfôrço industrial no sentido de fornecer, ao mercado consumidor dêsses ácidos, outros, quer orgânicos quer não, que tivessem paladar apropriado para o consumo como alimento e que fossem destituídos de toxicidade nas quantidades utilizadas habitualmente pelo homem. Dois caminhos apresentavam-se então : 1.º) — extrair ou sintetizar ácidos comumente presentes em alimentos naturais ; 2.º) — procurar obter outros agentes acidificantes não presentes usualmente nos frutos comestíveis e destituídos de toxicidade. Verdade é que muitos ácidos orgânicos existentes, abundantemente, nos frutos ou outras partes comestíveis do vegetal são tóxicos aos animais, acarretando mesmo efeitos nocivos pronunciados, como é o caso do ácido oxálico.

Um ácido encontrado, abundantemente, nos frutos do gênero *Pirus* é o ácido málico, cuidadosamente estudado quanto à sua industrialização por via sintética, já que seus efeitos eram mais ou menos conhecidos. Temos hoje, ao lado de pequenas quantidades disponíveis de ácido L. málico natural, o ácido D L málico sintetizado em grandes quantidades. Também o ácido glicólico, contido em muitos frutos, foi proposto, desde a segunda grande guerra, como agente acidificante, pois que sua preparação por via sintética é fàcilmente exeqüível a partir do ácido acético.

O ácido lático, outro dos que ocorrem em muitos frutos e órgãos animais, encontrou enorme aplicação como agente acidificante em alimentos. Deve-se a difusão do seu uso à manufatura econômicamente realizada por fermentação da sacarose.

No campo econômico, enormes progressos foram feitos com a produção de ácido cítrico por via fermentativa do açúcar, levando a que tal ácido tivesse consumo grandemente aumentado.

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Por fim, entre os ácidos orgânicos que têm sido propostos e bem aceitos, destaca-se o ácido glicônico, fornecido comercialmente na forma de solução aquosa xaroposa. Êste ácido é encontrado também em muitos frutos, sendo fàcilmente preparado pela fermentação oxidativa da glicose. O ácido em si, bem como seus sais (de Ca, Fe, Cu), é extensivamente aplicado na terapêutica moderna de sais minerais.

O único ácido proposto como agente acidificante de alimentos, não orgânico, foi o ácido fosfórico. Êste, bem como os demais ácidos enumerados, tem sabor ácido agradável e é tido como atóxico nas quantidades usadas, apresentando, além disso, a vantagem de poder ser fabricado em alto grau de pureza.

A primeira questão que se levanta a tôda e qualquer proposição de um novo agente acidificante utilizado na alimentação é saber o grau de toxicidade dêsse novo agente, desde que outras propriedades sejam favoráveis ao seu uso na alimentação, como sejam grau de pureza, sabor, aroma, etc.

Os dados de toxicidade encontrados na literatura científica foram reunidos no presente trabalho, sempre que possível ao lado de outras informações de interêsse farmacológico. Ácidos há que foram minuciosamente estudados em diversos animais de laboratório, quanto a seus efeitos, toxicidade aguda ou crônica, doses letais, etc. Outros, porém, apesar de muito conhecidos, não foram convenientemente abordados sob êsse prisma.

Passaremos, a seguir, a examinar, isoladamente, cada ácido.

ÁCIDO CÍTRICO

A literatura científica registra trabalhos que, por vêzes, depoem contra o uso indiscriminado dêsse ácido.

ZIPKIN (1947), do Instituto Nacional de Saúde de Maryland, estudando o conteúdo de ácido cítrico na saliva humana, revelou que, no homem adulto, cada 100 cm³ de saliva contém de 0,6 a 2,0 mg de ácido cítrico. Zipkin sugere existir uma relação entre o ácido cítrico salivar e a erosão e cárie dentárias. A descalcificação do tecido dentário pelo ion cítrico é sugerida pela observação de que êste ion forma com o de cálcio um complexo solúvel e fracamente ionizado, segundo SHEAR, KRAMER e RESNIKOFF (1929). Mc CLURE e RUZICKA (1946) observaram, em laboratório, que o citrato, em bebida pràticamente neutra, tem pronunciada ação destrutiva sôbre o tecido dentário "in vivo".

Em contraposição aos achados de STAFNE e LOVESTEDT (1947), NEWMAN (1948), verificou que não se observa nenhuma evidência de que a destruição de dentes possa ser atribuída aos efeitos do ácido cítrico de frutas cítricas. Experiências foram executadas entre portorriquenhos que consomem, em média, 4,24 laranjas ou "grapefruit" por dia, durante 5 a 6 meses do ano.

LESCHKE (1932) relata que, como efeito impediente da circulação sangüínea, o ácido cítrico assemelha-se bastante ao ácido oxálico, levando, ocasionalmente, a intoxicações graves e até mesmo letais. É particularmente grave em crianças, nas quais, após a ingestão de mesmo 1 a 2 limões, podem ocorrer deficiências de circulação e desmaios. Menciona o autor o caso

de uma jovem que ingeriu, de uma só vez, 25 g de ácido cítrico para efeito laxante ; apesar de ter vomitado abundantemente, veio a falecer.

Quanto ao destino do ácido cítrico ingerido, SHERMAN, MENDEL e SMITH (1936) realizaram estudos em animais de laboratório, observando que o cão tem a possibilidade de destruir grandes doses de ácido cítrico. Pela administração oral de 0,5 a 2,0 g do ácido por quilo de animal, cêrca de 0,7% não é oxidado, aparecendo na urina. A ingestão daquele ácido não afetou nem o pH nem o nitrogênio total na urina coletada em 24 horas. No homem, KUIPER e MATTILL (1933) concluem que o ácido cítrico, quando ingerido de 2 a 20 g, é ràpidamente oxidado no organismo, restando sòmente 1,5 a 2,5%, que é excretado pela urina.

O efeito tóxico do ácido cítrico e de seus sais foi, recentemente, determinado por GRUBER Jr. e HALBEISEN (1948). Verificaram êsses autores que os sintomas provocados pela administração de grandes doses de ácido cítrico e citratos assemelham-se aos da deficiência de ion de cálcio, isto é, aumenta a atividade geral, aparece hiperpnéia, vaso-dilatação periférica, salivação, convulsões clônicas e tônicas, cianose e, às vêzes, morte. A dose letal média, em milimoles por quilo de animal, foi determinada em animais diversos. Os resultados estão resumidos na tabela abaixo :

	D.L.$_{50}$: milimoles/quilo				
	Coelhos intravenosa	Camundongos intravenosa	Camundongos intraperit.	Ratos intraperit.	Coelhos intravenosa
Ácido cítrico ...	1,72	0,22	5,0	4,6	0,331
Citrato de sódio	1,76	0,23	7,6	6,3	
Citrato bissódico	1,77	0,30	7,5	7,3	
Citrato trissódico	1,74	0,60	5,5	6,0	

Se tomarmos o resultado das D.L.$_{50}$ para o coelho, por via intravenosa, e o transferirmos para o homem de pêso médio (70 quilos), teremos então a D.L.$_{50}$ = 7,1 g por 70 quilos. *

A toxicidade crônica, após prolongada adminstração do ácido cítrico foi determinada por KROP e GOLD (1945), revelando que, na dose de 1,38 g do ácido por quilo administrado a cães, durante 112 a 120 dias, num nível de 103 a 108 doses (3 cães), êstes animais não apresentavam nenhum sintoma de intoxicação, mesmo no exame histológico. Tal resultado, calculado para o homem de pêso médio de 70 quilos, dar-nos-ia, como dose não tóxica, cêrca de 63,6 g diárias.

Sabemos, pelo trabalho de Kuiper e Mattill, já referido, que o ácido cítrico, ingerido numa dose de 2 a 20 g, é ràpidamente metabolizado no organismo e que sòmente 1,5 a 2,5% são excretados na urina. WEISS, DOWNS e CORSON (1923) fixaram em 2,5 g por quilo, em coelhos, a dose tolerável

* Tôdas as doses para o homem foram calculadas a partir das doses conhecidas, em animais de laboratório, utilizando-se a fórmula de Mech.

N... NDALE — The Extra-Pharmacopeia. London, Pharmaceutical Press, 1941 ; p. 32.

de ácidos málico e cítrico para administração oral prolongada e ao redor de 2 g por quilo a dose de ácido tartárico. Êsses dados, transferidos para o homem de 70 quilos, nos conduzem às doses diárias que podem ser ingeridas durante longo tempo, sem inconveniente algum para a saúde :

Ácido cítrico e málico — 53 g/70 quilos
Ácido tartárico — 43 g/70 quilos

ÁCIDO TARTÁRICO

De um modo geral, concordam os farmacologistas em que o ácido tartárico é pouco mais tóxico do que o ácido cítrico. Fato curioso, entretanto, é que, dentre todos os ácidos utilizados presentemente como acidificantes, em alimentos, é o ácido tartárico o único dotado de certa atividade antibacteriana. Esta atividade, não só a exerce o ácido livre, como também muitos de seus sais, como seja, por exemplo, o bitartarato de potássio (SEELING e colab., 1943).

Já VIOLLE (1937) havia verificado que uma solução a 0,35% do ácido tartárico em água destilada destruia *E. typhosa* em 20 ou 30 minutos, *B. dysenteriæ* Shiga em 2 horas e *cholera* em 10 minutos. Verificou mais que uma solução a 0,4% faz *E. coli* diminuir em número, mas, mesmo após 24 horas, permanece viva. FROSINI (1940) afirma que a água potável pode ser desinfetada com ácido tartárico ou cítrico. A desinfeção dá-se em 20 minutos em relação a *B. cholera* ; em ½ hora para *B. dysenteriæ* ; 3 horas para *B. typhoide*, sendo, no entretanto, o *B. coli* resistente à ação dêsse ácido.

Ainda sôbre a utilização do ácido tartárico como desinfetante da água potável, surgiram outros trabalhos, entre os quais os de NEGRO e BERTASSO (1940) e CAMBOSU (1940).

Sôbre o destino do ácido tartárico no corpo humano, DAKIN (1922) afirma que o ácido tartárico (formas D e L) é oxidado no corpo humano menos ràpidamente do que os ácidos málico e succínico. Segundo FINKLE (1933), sòmente parte do ácido tartárico administrado oralmente a pessoas, na dose de 2 g, é excretado inalterado (cêrca de 20%). Os restantes 80% são destruídos no trato intestinal pela ação de bactérias, segundo afirmam UNDERHILL e colab. (1931). Êsse trabalho de Underhill reforça os achados de PICKENS e HETLER (1930) citados por Finkle. Pickens e Hetler verificaram que, pela adminstração de grandes doses de suco de uvas a pessoas, a urina se tornava mais ácida e continha ácido tartárico livre.

Experiências realizadas por UNDERHILL e colab. (1931) indicaram que nem o cão nem a cobaia ou o coelho podem utilizar o ácido tartárico.

KROP e GOLD (1945) estudaram a toxicidade dos ácidos tartárico, cítrico e glicólico e respectivo sal sódico, após prolongada administração em cães ; verificaram que a ingestão, durante 112 a 114 dias (100 a 103 doses), de 0,9 g/quilo de ácido tartárico (equivalente a 0,5 g/quilo de ácido glicólico) provocara a morte a um, já depois de 90 dias, com azotemia. Outros três cães apresentaram teores normais quantitativos dos componentes do sangue, se bem que houvesse alterações no que diz respeito à urina. O exame histológico revelou, no cão morto após 90 dias, degeneração tubular no rim. Portanto, na dose de 0,9 g/quilo, no cão, o ácido tartárico p... r nefrotó-

xico. O estudo do efeito da ingestão de ácido tartárico e outros ácidos em ratos foi efetuado por FITZHUG e NELSON (1947). Vinte ratos, entre machos e fêmeas, receberam dietas contendo 1,2% de ácido tartárico. Em todos os animais de experiência observou-se ganho de pêso, após 52 semanas de observação, sem que fosse notada alguma alteração parenquimatosa nos órgãos. (Pêso médio dos ratos M = 437,8 ; F = 258,0g). Isto significa que ratos pesando 258 g receberam, durante aquêle período de tempo, alimentação diária contendo 1,2% de ácido, sem revelar algum sintoma de intoxicação. Dêsses dados depreende-se que, se um rato se alimentar, diàriamente, com 50 g de alimento, (*ad libitum*), estará ingerindo 0,6 g do ácido tartárico — para 250 g, digamos, de pêso do animal. Isto dará cêrca de 2,4g/quilo de animal.

ÁCIDO LÁTICO

VLADESCO (1936) verificou que a saliva de indivíduos normais continha, por 100 cm³, ácido lático nas quantidades de 3,98, 4,24 até 13,61 mg ; no cão encontrou 4,39 mg por 100 cm³ de saliva.

NEUWIRTH e KLOSTERMAN (1940) verificaram, experimentalmente, que, após estarem 10 minutos na cavidade bucal pedaços de banana, sacarose, glicose ou amido, podia ser provada a formação de ácido lático por ação de bactérias. A saliva filtrada em filtros Seitz não mostrava tal ação. Por outro lado, julga SCHMITZ (1943) que a corrosão dos dentes seja devida principalmente ao ácido lático formado pela fermentação bacteriana na cavidade bucal.

NORPOTH e KADEN (1933) demonstraram que o suco gástrico contém de 6 a 25 mg de ácido lático por cento. Em gastrites, êsse valor é muito aumentado. Um aumento no sangue, por injeção de adrenalina, não correspondia a um acréscimo do conteúdo de ácido lático estomacal.

Estudos em animais de laboratório, sôbre a toxicidade dêste ácido, revelaram que êle é muito pouco tóxico. FÜRTH e ENGEL (1930) verificaram que coelhos podem receber, subcutâneamente, doses de 2,8 a 3,6 g de ácido lático por quilo, sem sofrer qualquer dano. Morrem, porém, quando alimentados com 1,6 g/quilo ou 0,6 g/quilo durante 3 dias.

Encontram-se, na literatura científica, algumas raras observações, que relataremos a seguir, por apresentarem certo interêsse elucidativo.

LECOQ (1936) reproduziu crises polinevríticas em pombos, idênticas às de avitaminose B_1, dando aos animais ração diária contendo 10% de ácido lático. Esta polinevrite aviária, causada pelo ácido lático, dá-se em presença ou ausência de glicídios, ou se no regime alimentar predominarem protídios ou lipídios. Em tal circunstância, o animal é impedido de utilizar a vitamina B_1.

Envenenamento por ácido lático com êxito letal foi comunicado por Führer, citado por LESCHKE (1932). Uma mulher de 27 anos recebeu, por engano, no duodeno, em lugar de 100cm³ de solução de sulfato de magnésio, uma solução de ácido lático a 33%, apresentando, então, vômitos com sangue, durante meia hora, e fortes dores. Houve hemoglobinúria, mas não hematúria. O pulso caiu. A morte deu-se após 12 horas. A necrópsia revelou [illegible]tação em 23 cm de comprimento do duodeno.

Já DEFREN (1920) havia discutido e recomendado o uso do ácido lático refinado em cervejas não alcoólicas e como substituto do ácido cítrico e o tartárico, em refrescos, balas, na indústria do pão, pós de fermento, geléias, vinagres, gelatinas e na conservação de carnes e produtos derivados de peixes.

BORDAS (1927), numa discussão sôbre a permissão do uso do ácido lático em alimentos, concluiu não haver objeção alguma ao seu uso em limonadas (refrescos), picles ou vinagres, desde que fosse puro, isto é, livre de cobre, zinco, arsênico, ácido oxálico e ácido sulfúrico.

O trabalho mais importante sôbre toxicidade do ácido lático e seu emprêgo em alimentos foi elaborado por COLLAZO e colab. (1932). Êsses autores dão um pequeno apanhado sôbre o que foi feito no assunto e passam, em seguida, a apresentar seus próprios resultados. O ácido lático, introduzido no intestino (leites ácidos, queijos, frutas, etc.) é absorvido e passa à circulação, ou se transforma em outras substâncias, ou ainda é eliminado. Administrado a coelhos por via oral, na quantidade de 1,6 g/quilo, demonstraram os mesmos perfeita tolerância, tendo, entretanto, lactocidemia e glicemia em 30 minutos.

Segundo Parnas (citado por Puyal), a toxicidade para coelhos, por via subcutânea, oscila entre 2,8 e 3,6 g/quilo, enquanto que, por via oral, Fürth e Engel (citados por Puyal) indicam a dose tóxica de 1,6 g/quilo, doses essas repetidas durante 3 dias. Os ratos sobrevivem à injeção subcutânea de 2 a 4 g/quilo e não apresentam qualquer manifestação de intolerância à administração por via oral de 2 g/quilo, durante longo tempo. Segundo os mesmos autores, no homem e no coelho, cêrca de 20 a 30% são eliminados na urina, quando ingerido na forma de limonadas ricas em ácido lático. No cão, se dado por via endovenosa, aparece na urina, em proporções variáveis ; sendo, porém, administrado oralmente, quase não é eliminado.

Bateherwick e Long (citados por Puyal) deduziram, pelo aumento da acidez fosfática da urina, depois da ingestão de leite ácido a 29°, que o ácido lático é completamente utilizado pelo organismo.

A administração por sonda de 0,6 a 1,6 g de ácido lático a cães de 8 quilos, prolongada durante 42 dias, foi perfeitamente tolerada pelos mesmos. Em coelhos, 5 g/quilo, dados em dias alternados, não evidenciaram manifestações tóxicas. Stauton, Faust, Parfentiew, Sentzeff e Sokoloff (citados por Puyal) indicam como doses máximas, em coelhos, toleradas as seguintes :

via endovenosa — 0,5 g
via subcutânea — 3,0 g } por quilo
via oral — 6,0 g

Fasold (citado por Puyal), de seus estudos de tolerância de ácido lático por crianças, chegou à conclusão, segundo suas experiências nas lactantes, de que se poderá administrar aos adultos de 70 quilos enorme dose de 200 g de ácido lático, sem inconveniente algum.

Se fixarmos como dose máxima tolerada 5 g/quilo, achado de Puyal e colaboradores, e se calcularmos para o homem de 70 quilos, [illegible]os a dose máxima tolerável de mais de 107 g de ácido lático. Se, de ou[illegible]o, tomar-

mos o resultado da observação da administração prolongada em cães, isto é, 1,6 g/8 quilos e transferirmos para o homem de 70 quilos, teremos, como dose tolerável, durante mais de um mês, a quantidade diária de 6,8 g de ácido lático, ou, em dias alternados (do coelho 5 g/quilo), cêrca de 107 g.

ÁCIDO MÁLICO

UNDERHILL e PACK (1925) estudaram o comportamento farmacológico do ácido málico e seus sais. Fixaram êsses autores a dose letal subcutânea no rato em 3 g/quilo, quando injetado como malato de sódio (a de tartarato de sódio é 3 g/quilo).

Na dose de 1 g/quilo, o malato de sódio é destituído de efeito tóxico para cães.

O ácido málico não é nefrotóxico e isto se deve ao fato de ser êle fàcilmente oxidado no organismo. Nas quantidades aplicadas para fins acidulantes, o ácido málico e seus sais podem ser tidos como destituídos de toxicidade.

KRANTZ Jr. e colab. (1931) observaram que o malato de sódio faz decrescer a concentração dos ions de hidrogênio do suco gástrico e aconselham que se faça a substituição do cloreto de sódio pelo malato de sódio, como condimento, porque, assim, decrescerá a acidez livre do suco gástrico, reduzindo-se a fonte de ácido clorídrico.

WEISS e colab. (1923) determinaram a toxicidade do ácido málico e o efeito cumulativo dêle e de outros ácidos em coelhos.

Onta (citado pelos últimos autores) verificou que o ácido málico, administrado a coelhos e cães, aparece na urina. Fornecido, oralmente, em grandes quantidades, é tóxico. De 10 a 20 g "per os" a coelhos, é inteiramente destruído. Quando a dose alcança 25 g, cêrca de 5% são excretados na urina.

WISE (1916) relata que, da dose não tóxica de 1g/quilo para o coelho, cêrca de 2 a 21% são excretados na urina. Já com dose de 3.3 g/quilo, aparecem sintomas tóxicos.

WEISS e colab. (1923) verificaram, em experiências sôbre 40 coelhos, que a dose letal do ácido málico é de 7 g/quilo. Os animais receberam soluções do ácido diretamente no estômago.

A tolerância a doses acumulativas foi observada em outros lotes de coelhos: 3 coelhos foram alimentados com 2,5g/quilo, durante 5 dias consecutivos, por 2 semanas: — 2 sobreviveram e um morreu após a primeira semana. Os sobreviventes receberam doses de 6 g/quilo. Um morreu. Ao outro, após 2 dias, foram dados 8 g/quilo, depois do que, morreu.

A dose tolerada na administração prolongada de ácido málico deve estar em redor de 2,5 g/quilo, ou, talvez, 2 g/quilo.

Calculando êsses achados para um homem de 70 quilos, teremos: dose letal — 150 g/70 quilos e dose diária tolerada na administração prolongada — 43 g/70 quilos.

Êsses resultados são quase iguais aos do ácido cítrico e superiores ao do ácido tartárico.

ÁCIDO GLICÓLICO

Constituinte normal do caldo de cana, caldo de beterraba e de uvas, assim como de muitos frutos.

Durante a segunda guerra mundial, foi tentada a introdução dêsse ácido como acidulante de alimentos, principalmente porque êle pode ser fabricado industrialmente por via sintética a preço compensador.

O ácido glicólico, introduzido no organismo, segundo experimentações de BARNES e LERNER (1943), não contribui para a formação do glicogênio no fígado. Também não causa formação de corpos cetônicos no sangue ou na urina. O ácido acético, ao contrário, na mesma concentração, é cetogênico.

O ácido glicólico é considerado como intermediário no metabolismo da glicina e da glicose.

GRIFFITH (1930) observou que a retardação do crescimento de animais, quando se dão grandes doses de ácido benzóico, é sustada ao ser administrado ácido glicólico.

A toxicidade do ácido glicólico, após prolongada administração, foi estudada por KROP e GOLD (1945). De seu trabalho resultou a evidência que o ácido glicólico na dose de 0,5 g/quilo diária, ao cão, não mostra nenhum efeito tóxico, por período que varia de 93 a 119 dias, com doses no número de 82 a 104. Um dêsses cães anteriormente tinha recebido 85 doses diárias de 0,194 g/quilo e, mesmo assim, nada de anormal ocorreu. O exame histológico dos órgãos revelou completa normalidade.

Êsses dados, transferidos para o homem de 70 quilos, conduzem a: 18 g/70 quilos, isto é, o homem poderá tolerar, perfeitamente, por longo tempo, uma dose diária de ácido glicólico, de cêrca de 18 g.

ÁCIDO GLICÔNICO

Existente em alguns frutos, proveniente da oxidação de açúcar correspondente, êste ácido pode ser preparado pela fermentação oxidativa da glicose. Seu sabor agradável e sua baixa toxicidade muito têm contribuído para a sua introdução na indústria alimentícia como acidulante em geral.

HERMANN e ZENTNER (1938) haviam observado que o ácido glicônico, bem como o ácido lático, administrados por via oral, são pouco excretados na urina de coelhos. O pH da urina diminui com o ácido glicônico.

HERMANN (1930) observou que ácido glicônico, quando injetado intravenosamente, não acarreta queda da pressão sangüínea (ao contrário dos outros ácidos orgânicos), nem influi no metabolismo do cálcio em coelhos e cães. Porém a administração parenteral causa um aumento de cálcio do sôro, isto é, mobiliza êste cation, o que vem explicar o fato que êle faz decrescer a toxicidade do ácido cítrico. Sòmente uma fração do ácido glicônico administrado oralmente é absorvido como tal e isto devido à decomposição por bactérias intestinais.

GAJATTO (1939) determinou como letal a dose de 7,63g/quilo do gliconato de sódio em coelhos, quando injetado intravenosamente; a morte do animal ocorre por ação depressiva exercida sôbre o sistema nervoso central.

Já para o ácido glicônico livre, HERMANN (1947) estabeleceu a dose letal de 85 cm³ de solução N/2 por quilo, administrado, intravenosamente, aos mesmos animais de experiência; isto corresponde a 8,3 g/quilo (em pêso, do ácido livre), isto é, pouco acima do achado de S. Gajatto para o gliconato de sódio. Se tomarmos, com dose tolerável, a décima parte dêsse valor, cêrca de 0,8 g/quilo, e se o transferirmos para o homem de 70 quilos, teremos a dose tolerável de cêrca de 17g de ácido glicônico, administrando-se por via oral. Doses únicas de 3 a 9 g, cada 2 horas, têm sido empregadas, por via oral, para abaixar o pH da urina, por SISK e TOENHART (1938), atingindo o mínimo de pH depois de 3-18 g de ácido glicônico em 2-3 horas. Resultados idênticos foram observados por GOLD e CIVIN (1939), sem, contudo, serem evidentes sintomas de intoxicação. Tais dados são bastante expressivos e bem falam a favor do uso do ácido glicônico como acidulante de alimentos, o qual, adicionado na quantidade de 0,2 ou 0,3%, estará, mesmo assim, bem longe dos limites de toxicidade.

ÁCIDO FUMÁRICO

Recentemente, foi êste ácido proposto como acidulante de alimentos e como droga medicinal. Êsse interêsse nasceu durante a segunda guerra mundial, nos Estados Unidos, em vista do decréscimo de importação de bitartarato de potássio, sub-produto na manufatura do vinho.

É conhecido que o ácido fumárico é integrante normal dos tecidos. Por outro lado, sua relação estrutural com o ácido tartárico e o fato dêle ser um sub-produto em sínteses microbiológicas levaram alguns pesquisadores a investigar o gráu de toxicidade do ácido livre e seus sais, bem como sua tolerância por administração prolongada. LEVEY e colab. (1946) realizaram estudos de toxicidade aguda e crônica do ácido fumárico, sendo que seus resultados podem ser assim resumidos:

a) Ratos alimentados com uma dieta sintética adequada, contendo 0,1 a 1,0% de ácido fumárico, não mostraram, por um a dois anos, anormalidade no crescimento, no conteúdo de hemoglobina, no número de eritrócitos e leucócitos, em dentes, cinzas de ossos, no fígado, nos rins, no estômago e no pâncreas.

b) Cobaias alimentadas com 1% de ácido fumárico, por um ano, não mostraram anormalidade no crescimento. A segunda geração, também tratada sob as mesmas condições desde o nascimento, não evidenciou efeitos tóxicos.

c) 75 indivíduos, que receberam 0,5 g de ácido fumárico durante um ano, não manifestaram conseqüências tóxicas.

d) Foi também pesquisado, em ratos, o efeito de grandes doses de ácido fumárico. A toxicidade oral do ácido fumárico é extremamente baixa; 7,04 g/quilo, por dia, não produzem efeito direto. Sob as mesmas condições, o ácido tartárico é mais tóxico.

A DL_{50} é, aproximadamente, igual a 8,0 g/quilo, oral.

A DL_{50} intraperitoneal é de cêrca de 0,587 g/quilo.

Dêsse trabalho, conclui-se que a dose letal no homem será, aproximadamente, de 80 g/70 quilos, quando administrado oralmente; por via intraperitoneal, a dose letal aproximada será de 5,8 g/70 quilos.

FITZHUGH e NELSON (1947) investigaram a toxicidade crônica de diversos ácidos, entre os quais o ácido fumárico. Mostraram êsses autores que ratos alimentados com dieta contendo 1,2 g% de ácido fumárico durante o ano não mostraram nenhum efeito tóxico. Revelou-se fracamente tóxico sòmente quando administrado ao alimento de ratos com 1,5 g% durante o ano. À base dêsse trabalho, os autores sugerem o uso do ácido fumárico em alimentos.

O fumarato de sódio, cuja DL_{50} é igual a 2,42 g/quilo, intraperitonealmente, em ratos tem sido proposto como catártico, em substituição aos sais de ácido cítrico, tartárico, glicônico, etc.

Maiores detalhes sôbre o assunto podem ser encontrados nos trabalhos de BODANSKY e colab. (1942), LOCKE e colab. (1942), GOLD e ZAHM (1943) e SMYTH e colab. (1945).

ÁCIDO FOSFÓRICO

Em relação aos outros ácidos já citados, é o ácido fosfórico o único que apresenta, em agrupamento indispensável aos organismos vivos, o ion fosfórico. Se não, vejamos: os ácidos cítrico, lático, glicônico e fumárico, introduzidos no organismo, são totalmente queimados; além disso, o próprio organismo sintetiza-os num metabolismo intermediário de glicídios e lipídios.

Os ácidos málico e tartárico não têm outro destino; são parcialmente eliminados pela urina e o restante entra em combustão orgânica, sendo, assim, destruídos. O mesmo, porém, não acontece com o ácido fosfórico; os fosfatos introduzidos no organismo não servem como substâncias energéticas, isto é, não fornecem energia calorífica pela sua combustão, mas sim tomam parte na constituição dos tecidos, especialmente do sistema de sustentação e da condutibilidade nervosa, ou integram a estrutura de enzimas (cocarboxilase, fermento amarelo) e nucleótidos, ou ainda entram no metabolismo intermediário de carboidratos (esteres de Embden, Cori, etc), ou no sangue, na forma de fosfato de sódio, contribuindo para a manutenção do pH sangüíneo. O organismo vivo não tem capacidade de sintetizar o ion fosfórico e a sua introdução no organismo é feita através da alimentação.

É conhecida a necessidade de fosfatos para os animais em crescimento, fosfatos êsses que, com o cálcio, entram na constituição dos ossos. A alimentação humana fornece, comumente, os fosfatos e o cálcio necessários ao organismo. Até hoje, não sabemos, ao certo, as necessidades do organismo infantil ou adulto em fósforo. O que sabemos, ao certo, é que o organismo humano elimina, pela urina, todo o excesso de fosfatos introduzidos nêle, acima de suas necessidades. De outro lado, um déficit de fosfatos no organismo não será compensado senão pela introdução de novos fosfatos.

Segundo von NOORDEN (1921), uma deficiência de fósforos observa-se, se bem que mais raramente, em casos de:

1.º — Excesso de trabalho muscular e, provàvelmente, outras atividades protoplasmáticas.

2.º — Desnutrição geral.

3.º — Por dieta que, sendo altamente calórica, seja deficiente em fosfatos.

Sabemos que o excesso de trabalho muscular provoca uma grande eliminação de fosfatos na urina.

De outro lado, encontram-se, na literatura, trabalhos de autores diversos, em que se verificou que a administração de fosfato de sódio (bifosfato de sódio), chamado Recresal, aos indivíduos, não só diminuiu o cansaço muscular, como aumentou, nesses indivíduos, a eficiência mental e física : — GRIESBACH (1928), POPPELREUTER (1930 e 1930a) RHAM (1932), CORBIAU (1936), GAJATTO (1939) e GREENFIELD (1941).

A alimentação mal orientada de indivíduos pode levá-los a uma carência de fósforo ; apesar dêsses indivíduos ingerirem alimentos ricos em cálcio, as quantidades de fosfato e cálcio não são suficientes para a formação do fosfato tricálcico e, conseqüentemente, não se fará o depósito ósseo, o que levará o indivíduo (em crescimento), independente de outras causas, ao raquitismo, além de outras possíveis perturbações de ordem nervosa e metabólica.

Von Noorden e Solomon (citado por von Noorden) haviam admitido que os fosfatos, introduzidos no organismo em alimentos (lecitinas, cefalinas, etc), eindem-se no estômago, formando o ion livre do ácido fosfórico.

Embden, experimentalmente, provou a necessidade do ácido fosfórico livre para processar-se a síntese e degradação do glicogênio, ligando-se à glicose para formar, intermediàriamente, esteres fosfóricos da glicose (ester de Embden, Cori e Harden-Young).

Apesar de se reconhecerem estas verdades científicas já há muitos anos, o uso do ácido fosfórico como medicamento e em alimentos encontrou uma certa repulsa. Terminou, por fim, pela demonstração experimental da baixa toxicidade, quer aguda, quer crônica, dêsse ácido.

É claro que o ácido fosfórico não poderá ser nunca administrado na forma de solução concentrada, pois, como tal, é cáustico e age sôbre mucosas, irritando-as, destruindo os tecidos e provocando enterites agudas. Mas tal fenômeno se verifica com qualquer ácido em forma concentrada, como no acima relatado, sôbre a morte de uma senhora a quem foi administrada, por engano, a dose de 100 cm³ de ácido lático a 33%. A necrópsia revelou irritação da mucosa em alguns centímetros do duodeno. Mesmo soluções de cloreto de sódio concentrado, e isso é conhecimento clássico, são igualmente tóxicas.

Os trabalhos fundamentais do estudo da toxicidade crônica do ácido fosfórico foram feitos principalmente por pesquisadores franceses e, entre êsses destacam-se Cautru, Martinet e Joulie.

CAUTRU (1904) relata, em seu trabalho, que já em 1900, com Bardet e Brun, administraram, durante um mês e meio, a um pato, uma grama por dia de ácido fosfórico oficinal (36,4% de ácido fosfórico), sem terem verificado qualquer sintoma extranho. Sendo o animal sacrificado e seu fígado examinado, não revelou anormalidade, nem esteatose. Cautru, em 1903, apresentou, à Sociedade de Terapêutica, sua observação sôbre 3 cobaias, às quais foram, durante 3 meses, administrados de 0,5 a 1 grama diárias de ácido fosfórico. O pêso das cobaias era de 350 g. Uma cobaia morreu de acidente de gaiola ; as outras duas sacrificadas não revelaram nenhuma anormalidade nos cortes histológicos de seus fígados e rins. Mais outras duas cobaias, que prosseguiram ingerindo as mesmas doses diárias, durante 11 meses depois de sacrificadas, não mostraram degeneração parenquimatosa, estando íntegro o tecido hepático.

Essas doses, transferidas para o homem de 70 quilos, equivalem a 50 g de ácido fosfórico oficinal por dia, ou sejam 18,2 g de ácido fosfórico (H_3PO_4).

O mesmo Cautru efetuou experiências em cães. Tomou 2 dêsses animais, dos quais 1 testemunha ; ao outro, pesando 8.500 g, administrou, durante um mês, sem qualquer inconveniente, de 1 a 2 g de ácido fosfórico oficinal (15 a 30 gôtas). A observação se prolongou durante um ano. Os dois cães (de 2 e meio meses de idade), que pesavam, o testemunha 8.800 g e o em experiência 8.500 g, foram submetidos ao mesmo regime alimentar (pão e carne cozida). Ao cão em experiência, deram-se, a mais, 15 gôtas (1g) de ácido fosfórico oficinal, diàriamente. Após um mês, o cão em experiência pesava ½ quilo a mais do que o testemunha. Nesse momento, a dose foi elevada a 30 gôtas (2g) do ácido fosfórico oficinal. Após mais de um mês, o pêso do cão em experiência era de 9.990 g e o do testemunha, 9.700 g.

Afim de verificar a resistência do animal em experiência, foi injetado 1 cm³ de cultura de bacilos de tuberculose na safena. Após 10 dias, passou-se a dar, ao cão em experiência, dose diária de 35 gôtas de ácido fosfórico oficinal (2,3g). Depois de 15 dias, os pesos dos cães eram : o em esperiência, 11.000 g e o testemunha, 11.050 g. Após um mês, o cão em experiência pesava 12.010 g e o testemunha, 11.300 g. Até êste momento (um mês após), não apareceu nenhum sinal de tuberculose. Aí foi inoculada a segunda dose de 1 cm³ de cultura de bacilos de tuberculose, subcutâneamente. O tratamento com ácido fosfórico continuou mais dois meses e meio. Decorrido êsse período, o cão em experiência pesava 21.200 g e não mostrava nenhum sintoma de tuberculose. Durante mais de 20 dias, o cão em experiência tomou de 100 a 200 gôtas de ácido fosfórico oficinal (cêrca de 10 g), ou seja 3,64 g de ácido fosfórico. No fim dêsse tratamento, o cão em experiência pesava 22 quilos e o testemunha, menos de 21 quilos. O cão descansou 17 dias; recebeu, depois, 300 gôtas de ácido fosfórico oficinal, sob forma mais concentrada ; o cão apresentou uma ligeira enterite, perda de apetite, etc. Após 10 dias, foi administrada dose menor (200 gôtas diárias) e os sintomas desapareceram. Após mais 10 dias, os pesos dos cães eram : o em experiência, 23.400 g e o testemunha, 21.500 g. Depois de 8 dias, foram injetados mais 1 cm³ de cultura de bacilos de tuberculose humana, em ambos os cães. O tratamento com ácido fosfórico, no cão em experiência, só foi suprimido depois de 12 dias decorridos dessa terceira inoculação de s da tuber-

culose. Os primeiros abcessos contendo bacilos de tuberculose desapareceram depois de 18 dias de inoculação. Ambos os cães emagreceram muito. Nesse dia (1 ano depois da experiência inicial), pesaram : o em experiência, 24.500 g e o testemunha, 24.000 g. O cão em experiência, que tomou ácido fosfórico durante 1 ano, apesar de ter recebido 2 inoculações, de 1 cm³ cada, de bacilos de tuberculose, não havia perdido sua resistência. Se tivesse esteatose hepática, êle não teria resistido à infecção bacilar.

Cautru fêz ainda outra série de experiências com 3 cães, sendo que um foi mantido como testemunha e os outros receberam, diàriamente, 5 g de ácido fosfórico oficinal cada um. Um dêsses cães foi sacrificado, após 4 meses de tratamento. Os cortes de fígado e rins mostraram-se normais. Dessas experiências, conclui Cautru, o ácido fosfórico não é um tóxico, nem esteatosante.

Após essas verificações, Jaulie, colaborador de Cautru, começou a empregar doses diárias de 200 a 400 gôtas (isto é, até 26 g de ácido oficinal), em doentes, para combater a diátese hipoácida e doses de 30 a 60 gôtas (cêrca de 2 a 4g) depois de 5 anos de idade. Jamais foi observado qualquer acidente. Em crianças de 18 meses, foram dadas 4 a 8 gôtas diárias (cêrca de 0,13 g de ácido fosfórico) ou mais e aos velhos, de 50 a 100 gôtas, isso é, até 2,3 g de ácido fosfórico, sem qualquer inconveniente.

Dessas concludentes experimentações, podemos tomar como dose diária tolerada por um cão de pêso médio, digamos 20 quilos, cêrca de 200 gôtas de ácido fosfórico oficinal, ou sejam 13,3 g, o que equivale a 4,74 g de ácido fosfórico. Transferindo êsse resultado para o homem de 70 quilos, teremos a dose de 11 g de ácido fosfórico, que poderá ser, sem inconveniente, dado ao organismo, diàriamente.

Se quisermos transferir a experiência de Jaulie para homens, teremos, como dose diária perfeitamente tolerável, cêrca de 26,6 g de ácido fosfórico oficinal, o que equivale a 9,48 g de ácido fosfórico.

Gueylard e Duval (1922) estudaram a influência da acidez e do radical ácido sôbre a toxicidade de ácidos diversos. Para isso, escolheram peixes como animais em experiência (Epinoches) e os ácidos fosfórico, lático, acético, propiônico, etc. Fizeram soluções dêsses ácidos com os correspondentes sais sódicos atóxicos N/50, que agem como tampão. No pH 2.8, os peixes sobreviveram 30 minutos em ácido fosfórico e 12 minutos em ácido lático. No pH 4,0, os peixes permaneceram vivos 8 horas em ácido fosfórico, 2 horas e 35 minutos em ácido lático e 11 minutos em ácido acético. Em pH 4,3, os peixes permaneceram vivos sòmente 9 minutos em ácido propiônico.

pH	Ácido Fosfórico	Ácido Lático	Ácido Acético	Ácido Propiônico
2,8	30 minutos	12 minutos	—	—
4,0	8 horas	2 hors. 35'	11 minutos	—
4,3	—	—	—	9 minutos

Portanto, no mesmo pH, a toxicidade dêsses ácidos cresce na seguinte ordem : ácido fosfórico, ácido lático, ácido acético, ácido propiônico.

A dose letal, em coelhos, por via intravenosa, determinada por Hermann (1947) é de 62 cm³ de solução N/2 por quilo, o que corresponde a 1,01 g/quilo de ácido fosfórico.

Para um homem de 70 quilos, a dose letal intravenosa será em redor de 22 g de ácido fosfórico.

Hoje em dia, o ácido fosfórico faz parte de quase tôdas as Farmacopéias : o Dispensatory Of The United States Of América (1947) indica seu uso direto no organismo humano. Não só em Farmacopéias, mas em Farmacologias e Tratados de Química Farmacêutica, encontramos estudos sôbre o ácido fosfórico. Veja-se, por exemplo, em Goodman e Gilman (1941), Sollmann (1944), Hager (1942), Reutter (1939) e Lebeau (1946), onde se lê que o ácido fosfórico diluído é aplicado como excitante da célula nervosa e utilizado também como acelerante da função gástrica, na medicação ácida em indivíduos com hipocloridria. Prescreve-se em solução, como xarope ou limonada em doses variáveis de 1 a 5 g, durante 24 horas, o que equivale de 100 mg a 500 mg de H PO.

Lecoq e Villuis (1932, 1932a e 1932b) e Lecoq e Villette (1933 e 1933a) estudaram a ação do ácido fosfórico e diversos derivados salinos sob o ponto de vista de um valor antirraquítico. De suas pesquisas, concluíram êsses autores que a atividade antirraquítica dos ortofosfatos tende a diminuir do sal mono ao trissódico. A eficiência dos sais ortofosfóricos está estreitamente ligada ao número de hidrogênios ácidos livres que o composto possui (Lecoq e Villuis, 1932).

Entre os diversos usos terapêuticos do ácido fosfórico, citam-se : para doentes com eliminação de saes minerais e hipoacidez urinária ; nos estados mórbidos em que o sistema nervoso deprimido perde sua resistência, neurastenias verdadeiras, em que há grande eliminação de fosfatos alcalinos pela urina ; nos casos de artritismos ; nos estados raquíticos (sífilis, impaludismo, etc) ; nos intoxicados (envenenamento por chumbo) ; em certos casos de dispepsias, etc.

Entre nós, Rolim (1942) tem aplicado solução de ácido fosfórico 1 : 200 em glauconas, injetando cêrca de 0,15 cm³. Como resumo, o autor descreve que o vítreo suporta bem soluções ácidas fracas de ácido fosfórico. Não foi observada complicação ou acidente, durante nem depois da acidificação vítrea.

DISCUSSÃO

O objetivo do presente trabalho foi trazer à luz a verdade científica acêrca da toxicidade dos ácidos mencionados, a fim de que sejam, os que ainda não o foram, admitidos como agentes acidulantes em alimentos e bebidas, entre nós.

O Decreto-lei 15.642 de 9/2/1946, que aprova o Regulamento do Policiamento da Alimentação Pública, admite apenas o uso dos ácidos cítrico, tartárico e lático como agentes acidulantes. À vista do que foi exposto, fácil é verificar que, com maior razão do que o ácido tartárico, se poderá incluir alguns dos outros.

Do relato feito e de acôrdo com os dados obtidos da literatura consultada, dados êsses resultantes de provas experimentais levadas a efeito com animais de laboratório, quer quanto à toxicidade aguda, quer quanto à crônica, podemos concluir o seguinte:

Doses diárias toleráveis para a administração prolongada, sem inconveniente para a saúde do adulto (70 quilos):

1.°) Ácido cítrico	—		53 g
2.°) Ácido málico	—		entre 43 e 53 g
3.°) Ácido lático	—		43 g em média
4.°) Ácido tartárico	—		43 g
5.°) Ácido fumárico	—	aprox.	43 g
6.°) Ácido glicônico	—		17 g
7.°) Ácido glicólico	—		18 g
8.°) Ácido fosfórico	—		11 g

RESUMO

Foi feito um apanhado da literatura científica, no que concerne à toxicidade dos ácidos cítrico, tartárico, málico, lático, fumárico, glicônico, glicólico e fosfórico.

A toxicidade crônica dêsses ácidos cresce na seguinte ordem:

Cítrico, málico, lático, tartárico, fumárico, glicônico, glicólico e fosfórico.

Todos os ácidos mencionados se mostram adequados para serem usados em alimentos e bebidas.

SUMMARY

In this paper, there is made a study of the scientific literature concerning the toxicity of the citric, tartaric, maleic, lactic, fumaric, gluconic, glycollic and phosphoric acids.

The chronic toxicity of these acids encreases in the following order:

citric, malic, lactic, tartaric, fumaric, gluconic, glycollic and phosphoric.

All the mentioned acids are proper to be used in food an drinks.

RÉSUMÉ

Dans ce travail, il est fait un abregé de la litterature cientifique sur la toxitée des acides citrique, tartrique, malique, lactique, fumarique, gluconique, glycolique et phosphorique.

La toxitée de ces acides croit dans l'ordre suivante: citrique, malique, lacti tartrique, fumarique, gluconique, glucolique et phosphorique
T acides peuvent être employés dans les aliments et les breuvage

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Taste Interrelationships. VI. Sucrose, Sodium Chloride, and Citric Acid in Canned Tomato Juice

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SUMMARY

Apparent sweetness, saltiness, and sourness in canned tomato juice was evaluated by trained judges categorized according to three levels of sensitivity. A method of paired-comparison constant-stimulus was used in which judges indicated the direction of the response as well as the magnitude of difference in taste intensity within each pair. The apparent sourness of citric acid was depressed by both sucrose and sodium chloride. The sweetness of sucrose was reduced by citric acid, but enhanced by low levels of sodium chloride. Although the saltiness of sodium chloride was reduced by sucrose, it was significantly enhanced by acid. In the latter combination of taste stimuli, variability between judges was exceptionally large. Frequency of reversal of direction of response between duplicate pairs was greater among judges of low than among judges of high sensitivity. The extent to which the results agree with previous relationships with aqueous media and lima bean purée is discussed.

INTRODUCTION

Extensive investigations have been made on the interaction of the basic tastes in solutions of distilled water (Fabian and Blum, 1943; Kamenetzky and Pilgrim, 1958; Beebe-Center *et al.*, 1959; Kamen *et al.*, 1961; Pangborn, 1960, 1961, 1962). Pangborn and Trabue (1964a) recently studied the taste interrelationship of sucrose, sodium chloride, and citric acid in lima bean purée and reported good agreement of responses with those established in distilled water.

In the present investigation, the main objective was to intercompare the apparent taste intensities of sucrose, sodium chloride, and citric acid in a natural food product which normally elicits sweetness, saltiness, and sourness—tomato juice. In addition, the effect of the original taste sensitivity of the experimental subjects on their subsequent responses was measured.

METHODS AND MATERIALS

Panel selection. A method of sequential analysis described by Amerine *et al.* (1959) was used to select 13 judges from 21 people tested. On each of 18 days of testing, each judge received 6 randomly presented triangle tests of commercially canned tomato juice, two varying in amount of added sucrose, two in amount of added sodium chloride, and two in amount of added citric acid. The judge's task was to designate the odd sample within each triangle set and to indicate whether the odd sample or the duplicates contained the greater intensity of the taste under study. The thirteen judges who fell within the region of acceptance showed a further separation into three distinct subgroups, which we will refer to subsequently as high (85.7–95.2% correct), medium (71.4–77.8%), and low (59.3–61.9%) sensitivity.

Experimental method. Used throughout the six phases of the investigation was a method of paired-comparison constant-stimulus described in detail previously (Pangborn, 1961). Each judge received four pairs in duplicate, for a total of eight randomly presented pairs per session. Within a pair, both members contained the secondary taste compound whereas only one contained the primary compound. Judges were asked to circle the number of the sample within each pair which contained the greater apparent intensity of the secondary taste sensation. In addition, the degree of difference within a pair was indicated on a scale labeled slight, moderate, large, or extreme. These descriptive terms were converted to integers of −1, −3, −5, and −7 if the sample without the primary compound was selected, and +1, +3, +5, and +7 if the sample with the primary compound was selected as more intense. These difference scores were then submitted to analysis of variance. Chi-square analysis was applied to determine the significance of difference in the paired selections.

Sample preparation and presentation. The tomato juice was prepared with no additives, from

Red Top variety tomatoes in the pilot plant of the Department of Food Science and Technology in September, 1961. The cans were stored at 40°F for 13 mo prior to initiation of the present study. The pH, total acidity, and refractive index of the juice were 4.4, 0.292%, and 1.3412. Throughout the investigation, pH, total acidity, and refractive index were measured for each experimental sample to assure accuracy of preparation. Concentrations of sucrose, sodium chloride, and citric acid were selected by thorough screening, to range from threshold to very intense for the specific combinations of tastes. The exact concentrations used are shown in Table 1 and in Figs. 1-6.

Samples were prepared on alternate days and stored in covered glass containers at 36°F. Reagent-grade sucrose and sodium chloride were added to the juice, w/v. The citric acid was first made into a 50% stock solution from which measured volumes were pipetted into measured volumes of juice. To compensate for dilution, equal quantities of distilled water (e.g. 1.7 ml water/1700 ml juice) were added to samples compared against those containing the citric acid.

The tomato juice was served daily between 9:00 and 10:00 A.M. at room temperature (70±2°F) under low, red illumination. Approximately 35 ml of sample was tested. Prior to tasting the experimental set, judges tasted a "warm-up" sample to orient them to the compounds being evaluated on that day. Instructions were given to refrain from swallowing any juice or distilled water, but to use distilled water for oral rinsing between pairs. Immediately after tasting, judges were provided with the results and a "reward" of cookies, crackers, or coffee cake.

Within a study, the order in which the treatments were served, the order of the pairs within a set, and the samples within a pair were randomized. Judges unable to attend a testing period were required to taste a "make-up" the following day, so that all samples were tested by all judges an equal number of times. As indicated in Table 5, only one judge was unable to complete each of the six studies. After a two-week break for the Christmas holidays, two days of reorientation were held before the presentation of experimental samples was resumed.

RESULTS

Study I. Effect of sucrose on apparent sourness of citric acid. The frequency of selection of the sample with the greater apparent sourness (Table 1) and the plotted difference scores (Fig. 1) show that sucrose depressed apparent sourness. The sourness of the higher levels of citric acid was depressed more than was the lowest level (0.01%). Analysis of variance of the difference scores

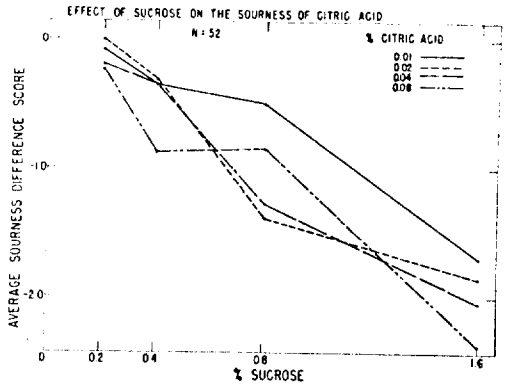

Fig. 1. Effect of sucrose on apparent sourness of citric acid in canned tomato juice.

showed no significant variation attributable to citric acid. The very highly significant difference between judges, between groups, and between judges within groups attests to the lack of agreement between individual judges in intensity of difference. Lack of significance due to replications and interactions involving replications emphasizes the reproducibility of response within the panel and within a judge. All three groups of judges agreed that sucrose depressed sourness, with judges 7 and 12 indicating slight positive average scores (Table 5).

Study II. Effect of citric acid on apparent sweetness of sucrose. Levels of citric acid between 0.04 and 0.16% significantly depressed the sweetness of 0.2 to 1.6% sucrose (Table 1, Fig. 2). Judges

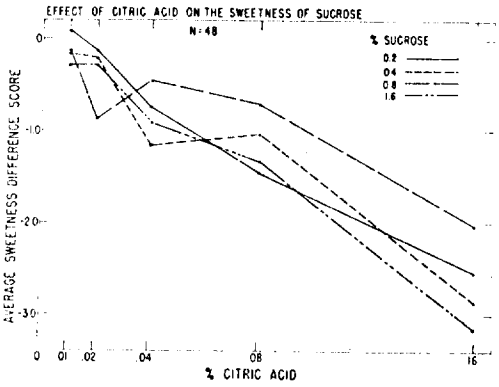

Fig. 2. Effect of citric acid on apparent sweetness of sucrose in canned tomato juice.

were in complete agreement on direction of response even though opinion of intensity of difference varied. Once again, judges and interactions involving judges were significant, whereas replications were not (Table 2). Groups of judges contributed significantly to the variation, with

Table 1. Taste intensity of sucrose, sodium chloride, and citric acid in canned tomato juice. Frequency of selection of sample without primary as most intense within each pair.

I. Sucrose-sourness (n = 52)
Primary:

Sucrose (%)	0.2	0.4	0.8	1.6	Total
% Citric					
0.01	26	32	36[b]	43[c]	137[c]
0.02	30	36[b]	43[c]	45[c]	154[c]
0.04	30	31	41[c]	46[c]	148[c]
0.08	30	40[c]	41[c]	49[c]	160[c]
Total	116	139[c]	161[c]	183[c]	599[c]

II. Citric-sweetness (n = 48)
Primary:

Citric acid (%)	0.01	0.02	0.04	0.08	0.16	Total
% Sucrose						
0.2	22	29	35[b]	41[c]	45[c]	172[c]
0.4	29	28	38[c]	40[c]	47[c]	182[c]
0.8	27	38[c]	31	35[b]	41[c]	172[c]
1.6	28	27	35[b]	37[c]	48[c]	175[c]
Total	106	122[c]	139[c]	153[c]	181[c]	701[c]

III. Sucrose-saltiness (n = 52)
Primary:

Sucrose (%)	0.3	0.6	1.2	2.4	Total
% NaCl					
0.2	32	29	33	38[c]	132[c]
0.4	26	33	31	37[b]	127[b]
0.8	33	36[b]	45[c]	44[c]	158[c]
1.6	34[a]	33	43[c]	44[c]	154[c]
Total	125[b]	131[c]	152[c]	163[c]	571[c]

IV. Sodium chloride-sweetness (n = 52)
Primary:

NaCl (%)	0.05	0.1	0.2	0.4	0.8	1.6	Total
% Sucrose							
0.2	11[c]	10[c]	5[c]	14[c]	21	31	92[c]
0.4	13[c]	6[c]	8[c]	12[c]	21	31	91[c]
0.8	19	11[c]	7[c]	8[c]	19	31	95[c]
1.6	12[c]	11[c]	8[c]	19	22	34[a]	106[c]
Total	55[c]	38[c]	28[c]	53[c]	83[b]	127[b]	384[c]

V. Citric-saltiness (n = 48)
Primary:

Citric acid (%)	0.015	0.045	0.135	Total
% NaCl				
0.16	20	19	13[b]	52[c]
0.48	29	17	5[c]	51[c]
1.44	17	11[c]	8[c]	36[c]
Total	66	47[c]	26[c]	139[c]

VI. Sodium chloride-sourness (n = 52)
Primary:

NaCl (%)	0.02	0.04	0.08	0.16	0.32	Total
% Citric						
0.01	28	39[c]	42[c]	39[c]	43[c]	191[c]
0.02	35[a]	33	44[c]	43[c]	42[c]	197[c]
0.04	36[b]	42[c]	41[c]	41[c]	45[c]	205[c]
0.08	26	35[a]	41[c]	39[c]	44[c]	185[c]
Total	125[b]	149[c]	168[c]	162[c]	174[c]	778[c]

[a, b, c] Significant difference within pairs at p = 0.05, 0.01, and 0.001 respectively.

Table 2. Analysis of variance of difference scores. Interaction of sucrose and citric acid.

Source of variation	I. Sucrose-sourness [a]		II. Citric-sweetness [b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(S) Sucrose	3	140.02***	3	5.85
(C) Citric acid	3	7.01	4	189.43***
(R) Replications	3	4.34	3	5.85
(J) Judges	12	31.06***	11	26.86***
(G) Groups	2	82.43***	2	32.64***
(Y) Judges within groups	10	20.78***	9	25.58***
S × C	9	3.67	12	5.42**
S × R	9	3.02	9	1.19
S × J	36	3.99	33	3.68*
G × S	6	1.11	6	3.04
Y × S	30	4.57*	27	3.83*
C × R	9	3.04	12	1.33
C × J	36	4.38*	44	8.73***
G × C	6	3.05	8	12.63***
Y × C	30	4.65*	36	7.86***
R × J	36	2.35	33	2.07
G × R	6	1.02	6	10.29***
Y × R	30	2.62	27	0.24
S × C × R	27	2.46	36	3.15
S × C × J	108	3.39	132	5.24**
S × R × J	108	2.47	99	3.72**
C × R × J	108	1.79	132	2.91
Remainder [c]	324	3.01	396	2.34

[a] Effect of sucrose on sourness of citric acid.
[b] Effect of citric acid on sweetness of sucrose.
[c] Error term (S×C×R×J).
*, **, *** Respectively significant at p = 0.05, 0.01, and 0.001.

Group I assigning the highest negative average difference score, followed by Groups II and III (Table 5).

Study III. Effect of sucrose on apparent saltiness of sodium chloride. With increasing additions of sucrose up to 2.4%, the saltiness of 0.8 and 1.6% sodium chloride was greatly depressed, whereas that of 0.2 and 0.4% was slightly lowered (Table 1, Fig. 3). That the saltiness of the different levels of sodium chloride was affected differently is verified by the analysis of variance (Table 3). Less variation was attributable to judges in this study than in the other five studies, for all but one judge assigned negative scores (Table 5). Neither replications nor groups of judges contributed significantly to the observed variation.

Study IV. Effect of sodium chloride on apparent sweetness of sucrose. Sodium chloride at concentrations of 0.05, 0.1, 0.2, 0.4, and 0.8% significantly enhanced the sweetness of all levels of sucrose (Table 1, Fig. 4). At 1.6% sodium chloride, however, apparent sweetness was significantly depressed. The analysis of variance shows no variation due to sucrose levels or replications, but very large variation due to salt levels and judges. Divergence of opinion between judges and between groups is readily seen in Table 5, where only Group II was in complete agreement and assigned the highest positive average difference scores.

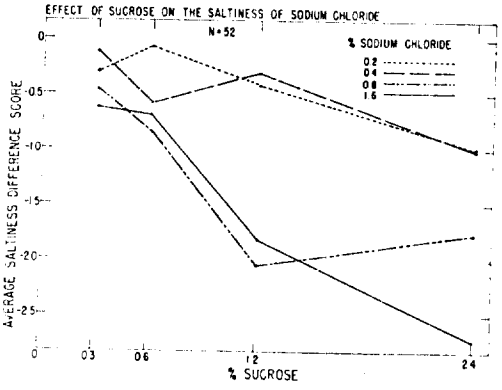

Fig. 3. Effect of sucrose on apparent saltiness of sodium chloride in canned tomato juice.

Study V. Effect of citric acid on apparent saltiness of sodium chloride. This study was the

Table 3. Analysis of variance of difference scores. Interaction of sucrose and sodium chloride.

Source of variation	III. Sucrose-saltiness[a]			IV. Sodium chloride-sweetness[b]		
	Degrees of freedom		Mean square	Degrees of freedom		Mean square
(S) Sucrose	3		70.02***	3		3.46
(N) NaCl	3		55.57***	5		315.19***
(R) Replications	3		1.72	3		2.50
(J) Judges	12		12.97***	12		232.76***
(G) Groups		2	2.13		2	318.60***
(Y) Judges within groups		10	15.14***		10	215.59***
S × N	9		9.57**	15		4.49**
S × R	9		0.65	9		4.08*
S × J	36		5.28*	36		4.74***
G × S		6	6.91		6	5.38**
Y × S		30	4.95		30	4.61***
N × R	9		1.45	15		3.46*
N × J	36		6.54**	60		27.57***
G × N		6	3.79		10	25.87***
Y × N		30	7.09**		50	27.91***
R × J	36		3.90	36		1.81
G × R		6	1.20		6	1.20
Y × R		30	4.44		30	1.93
S × N × R	27		4.64	45		2.04
S × N × J	108		5.26**	180		3.56***
S × R × J	108		3.10	108		1.94
N × R × J	108		2.33	180		2.23*
Remainder[c]	324		3.32	540		1.81

[a] Effect of sucrose on saltiness of sodium chloride.
[b] Effect of sodium chloride on sweetness of sucrose.
[c] Error term (S×N×R×J).
*, **, *** Respectively significant at p = 0.05, 0.01, and 0.001.

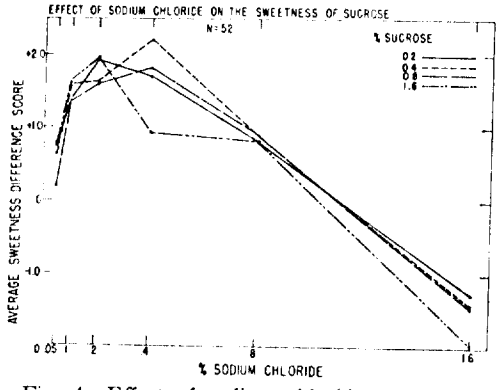

Fig. 4. Effect of sodium chloride on apparent sweetness of sucrose in canned tomato juice.

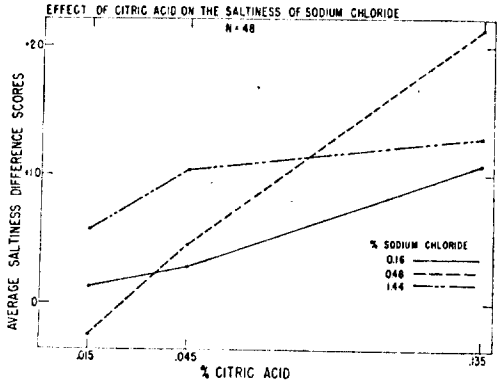

Fig. 5. Effect of citric acid on apparent saltiness of sodium chloride in canned tomato juice.

final one of the series, and, because of exhaustion of the tomato juice supply, it was necessary to use fewer concentrations. Note, however, that the range of concentrations selected for both the salt and the acid approximate the ranges used in the previous studies. Table 1 and Fig. 5 show that the acid enhanced the saltiness of the three levels of sodium chloride. Judges differed from each other slightly, but groups agreed on the pattern of general enhancement of apparent saltiness.

Study VI. Effect of sodium chloride on apparent sourness of citric acid. A picture of general depression of sourness by the addition of 0.02 to 0.32% sodium chloride is presented in Table 1

and in Fig. 6. All citric acid levels were affected similarly. Judges generally agreed on direction of response, except for a high positive value assigned by Judge 8 (Table 5). Again, replications and J × R were not significant (Table 4).

Influence of original sensitivity on reliability of response. As indicated previously, although all thirteen judges fell within the established range of acceptability on the basis of their sensitivity to sweetness, sourness, and saltiness in tomato juice, there were three sublevels of sensitivity among the thirteen judges. It was immediately apparent from the statistical analysis that these groups gave significantly different responses in all but two studies. Since the method requested an indication of apparent taste intensity, there were no "right" or "wrong" responses. Consequently the data were grouped according to the reproducibility of opinion of the three groups when judging duplicate pairs. In all six studies the frequency of reversal of direction of response within a set was higher for judges of the lower sensitivity. For the low-, medium-, and high-sensitivity subgroups, respectively testing 1680, 1564, and 2100 duplicate pairs, the percentage of reversals was 15.8, 15.2, and 8.8. Chi-square analysis showed that these percentages differed significately at $p = 0.001$.

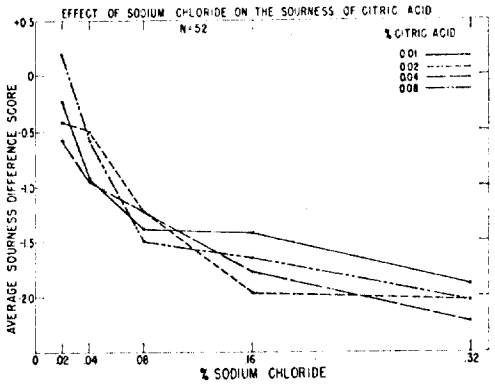

Fig. 6. Effect of sodium chloride on apparent sourness of citric acid in canned tomato juice.

DISCUSSION

Although different judges participated, the average taste intensity scores reported for

Table 4. Analysis of variance of difference scores. Interaction of citric acid and sodium chloride.

Source of variation	V. Citric-saltiness[a]		VI. Sodium chloride-sourness[b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(C) Citric acid	2	63.45***	3	2.38
(N) NaCl	2	6.86	4	108.84***
(R) Replications	3	1.96	3	1.40
(J) Judges	11	16.67***	12	109.38***
(G) Groups	2	1.30	2	132.44***
(Y) Judges within groups	9	20.09***	10	104.77***
C × N	4	12.51***	12	2.59
C × R	6	2.47	9	2.16
C × J	22	6.42***	36	4.12*
G × C	4	1.79	6	6.08*
Y × C	18	7.46***	30	3.73
N × R	6	6.18*	12	1.79
N × J	22	4.57**	48	12.40***
G × N	4	4.97	8	9.69***
Y × N	18	4.48*	40	12.94***
R × J	33	4.06*	36	2.85
G × R	6	3.48	6	3.03
Y × R	27	4.19*	30	2.81
C × N × R	12	1.82	36	2.25
C × N × J	44	2.98	144	2.45
C × R × J	66	3.27*	108	2.96
N × R × J	66	2.92	144	2.37
Remainder[c]	132	2.28	432	2.86

[a] Effect of citric acid on saltiness of sodium chloride.
[b] Effect of sodium chloride on sourness of citric acid.
[c] Error term (N×R×C×J).
*, **, *** Respectively significant at $p = 0.05$, 0.01, and 0.001.

Table 5. Average difference scores assigned by individual judges.[a]

Group[b]	Judge	Sex		I Sucrose-sourness	II Citric-sweetness	III Sucrose-saltiness	IV NaCl-sweetness	V Citric-saltiness	VI NaCl-sourness
			n	64	80	64	96	36	80
High	1	M		−0.94	−1.25	−1.44	−0.65	+0.78	−1.58
	2	F		−1.25	−0.85	−1.00	+2.06	+1.50	−1.58
	3	F		−1.19	−2.15	−1.59	−2.29	+0.67	−2.55
	4	F		−2.19	−1.43	−0.72	+0.33	−0.28	−1.98
	5	F		−1.72	−0.23	+0.03	+2.38	+0.56	−1.55
			$\bar{x}$	−1.46	−1.18	−0.94	+0.37	+0.64	−1.85
Medium	6	M		−0.50	−0.55	−0.66	+2.00	+0.78	−1.15
	7	M		+0.06	−1.43	−0.81	+1.92	+2.00	−2.45
	8	M		−0.41	....	−0.50	+1.04	....	+1.50
	9	M		−1.59	−1.78	−1.34	+2.54	−0.39	−1.55
			$\bar{x}$	−0.61	−1.25	−0.83	+1.88	+0.80	−0.91
Low	10	M		−0.66	−0.68	−0.63	+2.19	+1.33	−2.30
	11	M		−1.06	−0.83	−1.34	+0.58	+0.83	−0.55
	12	M		+0.16	−0.63	−1.16	−0.56	+0.78	+0.30
	13	F		−0.38	−0.50	−0.91	−1.08	+0.28	−0.35
			$\bar{x}$	−0.49	−0.66	−1.01	+0.28	+0.81	−0.73
Difference[c]				2.35	1.92	1.62	3.62	2.39	4.05

[a] Negative scores indicate the compound depressed apparent taste intensity. Positive scores indicate the compound enhanced apparent taste intensity.

[b] Grouped on the basis of sensitivity to sweetness, sourness and saltiness determined by triangular testing during original training period.

[c] Difference between highest and lowest value within each study.

the tomato juice are in excellent agreement with those for water solutions (Pangborn, 1961; 1962) and for lima bean purée (Pangborn and Trabue, 1964a). Individual judge variability was substantial in all studies, yet, in all media, sucrose and citric acid exhibited mutual masking effects, as did sucrose and sodium chloride, except for a slight enhancement of sweetness by low levels of sodium chloride. Citric acid enhanced all levels of sodium chloride whereas sodium chloride depressed the sourness of citric acid. The latter relationship was more clear-cut in the tomato juice than in the purée samples. There was no prior assurance that there would be agreement between the two media, for lima bean purée is a thick, starchy, bland product in which sweet and sour compounds occur at or below threshold levels, and saltiness is slight. Commercial tomato juice, in contrast, contains between 0.66 and 1.1% sodium chloride (Anderson *et al.*, 1954) and its sourness and sweetness are readily perceptible. Another variation was that of temperature of serving—165°F for the purée and 70°F for the tomato juice. To determine the influence of temperature on taste responses, it would be necessary to standardize the amount of solution ingested and the length of time it remains in the mouth. Although the rate with which the liquid passes over the tongue may be controlled, the temperature of the receptors may be of more importance than that of the stimulating substance.

It is generally recognized that experimental judges differ substantially in sensitivity and in reproducibility of sensory response, yet most investigators in food psychophysics ignore this source of variation by reporting mean values, only. In many cases, the variation is reported as the error term in the analysis of variance. Early in our studies on the interaction of the basic tastes we noted variation among judges in ability to detect differences, and in rating of taste

intensities; some judges consistently used the upper, and others the lower, part of the rating scale. Furthermore, with the experimental method used herein, the judge evaluates "apparent" sweetness, sourness, or saltiness. There is no right or wrong response, but between- and among-judge variability could occur as a result of shifting criteria of interpretation of these sensations in the presence of a secondary compound. In informal conversations with some judges, we noted a stated intention to achieve consistency of response. Some subjects decided during the training period that one compound depressed or enhanced apparent taste intensity, then attempted to maintain this direction of response throughout the study. As indicated by Jones and Marcus (1961), individual differences in judgment of taste stimuli may be ascribed to perceptual differences or to differences in habits of response.

In the present study, some judges remarked that the influence of the added compound altered the character of the secondary compound rather than the apparent intensity. If an appropriate method could be developed, a modification of the so-called time-intensity study (Neilson, 1958) could be used to establish the temporal and sequential changes in apparent taste intensity of both compounds in a mixture. The procedure would present some difficulties since the oral stimulation is relatively rapid.

Gregson and McCowen (1963) recently observed that some judges generally considered citric acid to increase sweetness in water solutions whereas other judges reported a decrease. Those investigators agree that it is misleading to treat all tasters as equivalents, and that judge effects should be separated in the analysis of the data.

In the present study, judges were selected on the basis of their ability to discriminate, then further subdivided into three levels of sensitivity. Results do not indicate that this original sensitivity influenced the direction of the response, i.e. whether the secondary compound enhanced or depressed the apparent intensity of the primary, but it was manifested somewhat in the magnitude of the difference scores (Table 5) and in the reproducibility of judgment. In retrospect, it would have been of interest to have retested the judges' discriminability at the termination of the study to determine whether the experience of the extended tasting influenced sensitivity.

Current investigations are showing that there is little relationship between the ability to detect a taste in the presence of additional compounds and the effect of one compound on the apparent intensity of another (Pangborn and Ough, 1964; Pangborn and Trabue, 1964b). Once again, even these relationships are influenced by individual judge variability.

ACKNOWLEDGMENTS

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ENZYME MECHANISMS CONTROLLING SUBSTRATE SUPPLY IN CARDIAC MUSCLE

1967, 329-333

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Cardiac muscle may utilise a number of substrates in support of respiration. These include glucose, pyruvate and lactate from carbohydrate sources and free fatty acids or ketone bodies from lipid sources. The utilisation of these substrates may be shown *in vivo* in man and experimental animals with cardiac catheterisation or by *in vitro* perfusion. *In vivo* glucose, pyruvate and lactate may be derived from plasma pools of these substrates or from cardiac glycogen; free fatty acids and ketone bodies may be derived from plasma pools or (in the case of free fatty acids) they may possibly be derived from circulating chylomicrons or muscle glycerides (*e.g.*, see Bing, 1965; Randle *et al.*, 1966; Enser *et al.*, 1967; Carlsen *et al.*, 1961; Willebrands, 1964). Amino acids are apparently not readily utilised by cardiac muscle in support of respiration (Clarke, 1957; Hicks and Kerly, 1960; Williamson and Krebs, 1961).

In diabetes, in man and in experimental animals, cardiac muscle in common with other tissues oxidises glucose at a much lower rate. Respiration is maintained by the oxidation of fatty acids, which may be extracted from the plasma pool of non-esterified fatty acids at an increased rate (Bing *et al.*, 1958; Ungar *et al.*, 1955). The alloxan-diabetic rat heart, perfused *in vitro* with or without insulin, shows a similar impairment of glucose utilisation. The maintenance of respiration under these conditions has been attributed to the oxidation of fatty acids derived from muscle glycerides. In experiments which are summarised below direct evidence on this point has been obtained by measurement of the change in cardiac lipids during perfusion and from estimates of the relative rates of hydrolysis and re-synthesis of glycerides in rat heart during perfusion.

When cardiac muscle is provided with more than one respiratory substrate, competition can occur for the oxygen consumed. Such substrate competition may be readily demonstrated by *in vitro* perfusion. Thus fatty acids or ketone bodies may inhibit the oxidation of glucose and *vice versa*, but fatty acids or ketone bodies are apparently the preferred substrate (Williamson and Krebs, 1961; Shipp *et al.*, 1961). Earlier studies with the perfused rat heart have indicated that the oxidation of fatty acids and ketone bodies may impair the oxidation of glucose through inhibition of pyruvate dehydrogenase, phosphofructokinase and hexokinase (Randle *et al.*, 1966). The mechanisms proposed involve inhibition of pyruvate dehydrogenase by acetyl-CoA, which may accumulate as a result of fatty-acid or ketone-body oxidation; and inhibition of phosphofructokinase by citrate formed from acetyl-CoA. Similar mechanisms may operate in the diabetic heart, where inhibition of these enzymes and accumulation of acetyl-CoA and citrate has been demonstrated (Garland and Randle, 1964; Pogson and Randle, 1966; Bowman, 1966; Randle *et al.*, 1968). Inhibition of hexokinase in the diabetic heart, and in the normal heart during the respiration of fatty acids and ketone bodies, has been provisionally attributed to inhibition of hexokinase by glucose 6-phosphate (Crane and Sols, 1955), which may accumulate because of phosphofructokinase inhibition. Kinetic studies with rat heart phosphofructokinase have shown that citrate raises the K_m for fructose 6-phosphate without changing the V_{max} of the enzyme (Pogson and Randle, 1966). Consequently, the inhibition of phosphofructokinase by citrate in the cardiac muscle cell may involve mechanisms which limit the formation of hexose monophosphates. Inhibition of hexokinase by glucose 6-phosphate would appear to be of major

importance in this connexion and detailed kinetic studies have been made to define the quantitative importance of glucose 6-phosphate concentration in the control of rat-heart hexokinase

TRIGLYCERIDE SYNTHESIS AND BREAKDOWN IN THE CONTROL OF FATTY ACID OXIDATION IN RAT-HEART MUSCLE *IN VITRO*

In the normal rat heart, perfused with medium containing glucose and insulin, the oxidation of glucose may account for approximately 80 per cent of the oxygen consumption. In the diabetic heart the oxidation of glucose was depressed and it accounted for no more than 50 per cent of the oxygen consumed. In an earlier study it was suggested that the oxidation of fatty acids from the breakdown of muscle glycerides may account for the balance of respiration. Qualitative evidence in support of this conclusion was obtained from measurements of glycerol output (as a measure of lipolysis rate) and concentrations of free fatty acids and fatty acyl-CoA, which were increased in the diabetic tissue. The evidence was not entirely conclusive in the absence of quantitative data. In particular, the measurement of lipolysis rates, based on glycerol output, did not necessarily provide a quantitative estimate of the rate of fatty-acid oxidation in the absence of estimates of the rate of re-esterification of fatty acids. Moreover, the use of glycerol output as an index of lipolysis rate may be criticised because of the possibility of glycerol utilisation by the heart (Kreisberg, 1966; Robinson and Newsholme, 1967) and of glycerol formation from glycerol phosphate (Vaughan, 1961).

In more recent experiments, quantitative estimates of the rate of fatty-acid oxidation in the perfused heart have been obtained in two ways. Direct measurements of the loss of lipid during perfusion have been made in the diabetic heart. Estimates of the rate of re-esterification of fatty acids in the heart have been obtained from measurements of the incorporation of radioactivity from [U-^{14}C] glucose into glyceride glycerol and glycerol and of the specific activity of lactate produced by the heart (which was shown to be similar to the specific activity of glycerol phosphate). The rate of fatty-acid oxidation could thus be independently computed from the difference between glyceride hydrolysis

TABLE I

Contributions of glucose and glycogen and endogenous triglyceride to respiration in the perfused rat heart

	Normal heart	Alloxan-diabetic heart
Triglyceride loss (μM fatty acid/g dry muscle/hr)	—	41.6
Lipolysis rate (μM glycerol/g dry muscle/hr)	4	20
Re-esterification rate (μM glycerolphosphate esterified/g dry muscle/hr)	1.2	3.4
Difference (lipolysis — re-esterification) (as μM fatty acid/g dry muscle/hr)	8.4	49.8
Oxygen consumption (μM/g dry muscle/hr)	1800	1850–2000
Oxygen consumed in glucose oxidation (μM/g dry muscle/hr)	1482	1038
Oxygen consumed in fatty acid oxidation (μM/g dry muscle/hr):		
based on triglyceride loss	—	955
based on (lipolysis — re-esterification)	196	1150
based on (total oxygen consumption — oxygen utilised for glucose oxidation)	318	812–962

For details of methods used see Denton and Randle (1967*a*, *b*).

and glyceride synthesis. The methods have been described in detail elsewhere (Denton and Randle, 1967*a*, *b*) and the results may be summarised as follows below.

Experiments, in which normal hearts were perfused without added substrate for 60 minutes, showed that there was disappearance of glycerides (mono-, di- and triglycerides), but no significant change in phospholipid concentration. Since cardiac contraction ceased after 40 minutes, it appeared that glycerides, but not phospholipids, could be readily utilised in support of respiration. These results and conclusions are in general agreement with those of Olson and Hoeschen (1967).

The results of these measurements in normal and diabetic hearts are summarised in Table I. In the diabetic heart perfused with glucose and insulin, loss of glyceride during perfusion was detected by direct measurement. The rate of loss of glyceride was sufficient to account for the difference between the total oxygen consumption and the oxygen utilised by glucose oxidation. In the normal heart, the rate of re-esterification of fatty acid was less than the rate of lipolysis (as measured by glycerol output) and the difference gave a value for the rate of fatty-acid oxidation, which was consistent with the rates of oxygen consumption and glucose oxidation. In the diabetic heart, the rates of lipolysis and re-esterification were both markedly increased; and the difference gave a value for the rate of fatty-acid oxidation, which was consistent both with the measured loss of glyceride and with the oxygen available for fatty-acid oxidation. These results are consistent with the view that fatty-acid oxidation is accelerated in the diabetic heart *in vitro*; that the fatty acids are derived from the hydrolysis of muscle glycerides; and that the increased rate of fatty-acid oxidation depends upon an increased rate of lipolysis and not upon a diminished rate of re-esterification of fatty acids.

GLUCOSE 6-PHOSPHATE CONCENTRATION AND THE CONTROL OF GLUCOSE PHOSPHORYLATION IN RAT-HEART MUSCLE

The idea that glucose 6-phosphate concentration may control rates of glucose phosphorylation in rat heart originated in the observation that glucose 6-phosphate is an inhibitor of rat-heart hexokinase and that the rates of glucose phosphorylation are inversely related to the muscle concentration of glucose 6-phosphate (Crane and Sols, 1955; Newsholme and Randle, 1961, 1964; Regen *et al.*, 1964). The possibility of other mechanisms, such as a fall in the concentration of hexokinase in diabetes, as seen in adipose tissue (McLean *et al.*, 1966), or control by the concentrations of inorganic phosphate, ADP, and 5′AMP (Tiedermann and Born, 1959; Crane and Sols, 1955), has been also suggested. One approach to the control of hexokinase in rat heart appeared to be to predict, from the kinetic properties of the enzyme and the concentrations of enzyme, substrates, inhibitors and activators in the heart, the rates of glucose phosphorylation under different experimental conditions. The extent to which predicted rates and measured rates of glucose phosphorylation are correlated might thus provide information about the control of hexokinase. Details of the methods used and of the errors which may be introduced by assumptions about the distribution of hexokinase and its substrates and effectors in cardiac muscle have been given elsewhere (England and Randle, 1967).

Hexokinase in rat heart is found in both soluble and particulate fractions. The kinetics of the two fractions have been separately investigated. The K_m values for glucose and $MgATP^{2-}$ were virtually identical. Of a large number of muscle metabolites tested as effectors, glucose 6-phosphate, ADP, and 5′AMP were inhibitors. The inhibition by glucose 6-phosphate was partly dependent upon ATP concentration (mixed inhibition). ADP and 5′AMP inhibition was competitive with ATP. The soluble enzyme was more sensitive to inhibition by glucose 6-phosphate at physiological concentrations and the particulate enzyme was more sensitive to inhibition by ADP and 5′AMP. Inorganic phosphate at concentrations within the physiological range partially relieved glucose 6-phosphate inhibition; but the effect was not large at physiological glucose 6-phosphate concentrations.

The total activity of hexokinase in the normal heart was 45.0 units/g dry muscle at

37°C. There was no change in total activity in alloxan-diabetes. The total activity of hexokinase in heart extracts was much greater (× 5) than the activity expressed in the perfused heart. This suggested that the enzyme is inhibited in the cardiac muscle cell. Calculations based on the kinetic data for the enzyme and cell concentrations of substrates and effectors indicated that this inhibition was mainly due to glucose 6-phosphate (90%) with a small contribution from ADP and 5′AMP (10%). Such calculations enabled rates of glucose phosphorylation in the perfused heart under a variety of experimental conditions to be predicted. These were compared with rates of glucose phosphorylation measured accurately in a microcirculation perfusion apparatus, in which glucose uptake could be measured minute by minute. The agreement between the predicted and measured rates shown in Figure 1 was very close. These studies have involved a number

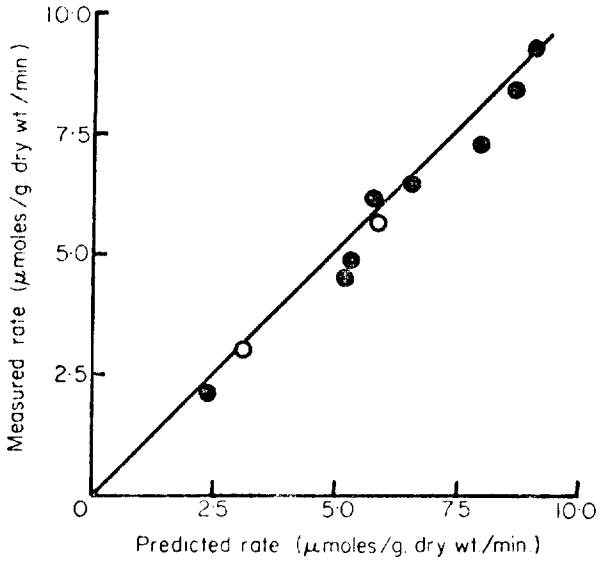

Fig. 1. Correlation between rates of glucose phosphorylation in rat heart predicted from the kinetic properties of rat-heart hexokinase and muscle concentrations of substrates and effectors and those measured in the perfused heart.

The line drawn is of 45° slope and represents absolute correlation; it is not necessarily the best fit for the experimental points. The closed circles are from data of England and Randle (1967) and the open circles are based on data given by Williamson (1965).

of assumptions, for which some experimental justification has been given (England and Randle, 1967). Thus it has been assumed that ATP in the cell is mainly present as $MgATP^{2-}$, that glucose 6-phosphate is confined to extramitochondrial water, and that this is 75% of the cell water, and that the proportions of soluble and particulate enzymes in the cell are those measured in extracts and that this does not vary under different experimental conditions.

GENERAL CONCLUSIONS

In an earlier study it was suggested that the oxidation of fatty acid accelerated in the perfused rat heart in alloxan-diabetes; that this is not a consequence of diminished

glucose utilisation, but is due to accelerated triglyceride hydrolysis; and that defects in the uptake and oxidation of glucose in the diabetic tissue might be a consequence of the increased rates of fatty-acid oxidation. The results of the present studies, which show that the breakdown of triglyceride in the diabetic tissue is sufficient to account for the rate of fatty-acid oxidation required, are consistent with this proposal. The measurement of rates of re-esterification of fatty acids in the heart has shown that this is increased in diabetes. This observation lends support to an earlier conclusion that the increased rate of fatty-acid oxidation in the diabetic tissue is caused by an increased rate of lipolysis. The enzyme mechanism for this change has yet to be defined.

The results of the present studies on the control of rat-heart hexokinase indicate that the concentration of glucose 6-phosphate is the major factor regulating glucose phosphorylation. The results are, therefore, consistent with the idea that inhibition of phosphofructokinase by citrate, in diabetes and during the oxidation of fatty acids and ketone bodies in the normal heart, is responsible for hexokinase inhibition. The control of glycolysis by citrate may, therefore, be regarded as a process of co-ordinated regulation of both phosphofructokinase and hexokinase.

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METABOLISM OF CITRIC ACID IN UROLITHIASIS[1]

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Increasing evidence implicates citric acid as a factor in the formation of urinary stones composed of calcium salts. The present paper is concerned with the urinary excretion of citrate in normal persons and in patients with stone. There have been no previous studies of blood and urinary citrate tolerance levels following citric acid administration in man.

Sabbatani[2] first observed that citrate bound calcium ions forming a soluble complex which is only slightly ionized. This phenomenon enables citrate salts to dissolve stones and bone in the test tube. Practical advantage has been taken of this effect by Albright, Sulkowitch and Chute[3] in the solution of urinary calculi by lavage with buffered citrate solutions. The dissociation of calcium citrate has been studied by Hastings and co-workers[4] and occurs as follows:

$$Ca_3\ Cit_2 \rightleftarrows Ca^{++} + 2\ Ca\ Cit^{-} \quad (1)$$

$$Ca\ Cit^{-} \rightleftarrows Ca^{++} + Cit^{=} \quad (2)$$

Thus, in the presence of citrate a soluble negatively charged complex is formed and the number of calcium ions is greatly reduced with consequently less chance to form insoluble calcium compounds.

Citric acid was first discovered in the animal organism by Soxhlet and Henkel[5] who found large quantities of it in cows' milk. Four body fluids contain large amounts of citrate; prostatic fluid, semen, milk and urine. A great concentration of citric acid in the animal organism occurs in human prostatic fluid[6] which may contain 2.6 gm. in 100 cc.[7] The skeleton[8] contains much larger amounts of citric acid than other tissues; the citrate content may be as much as 1.6 per cent of dry, fat free bone.

Amberg and McClure[9] first clearly demonstrated citrate in the urine of normal persons where it is excreted at times in large amounts (table 1). Citric acid in the urine occurs both from endogenous and exogenous metabolism. As proof of the endogenous origin is the fact that citrate still is excreted in the urine after a prolonged period on a citrate-low diet.[16]

The excretion of citric acid in the urine is influenced by several known factors.

1. *Age.* The citrate excretion of infants is low[10,11,12] compared with the urine of adults.

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2. *Urinary pH.* In an acid urine citric acid is usually decreased in amount and in an alkaline urine the value is increased.

In the acidosis of uremia[13] and diabetic coma[13,14] citrate excretion is markedly decreased. Östberg[13] discovered that oral ingestion of acid decreased the urinary citrate concentration; this may be brought about by the administration of hydrochloric acid[14], ammonium chloride[14,15] and other acid radicles.

Östberg[13] showed that ingestion of alkali increases the citrate content of urine; this occurs after intake of sodium bicarbonate[13,14,15] and other alkalies. Sherman, Mendel and Smith[11] observed that $NaHCO_3$ increased the citric acid output in the urine of man, dog and rat. Alkalosis resulting from hyperpnea increased urinary citrate[15].

3. *Diet.* There is an increased citrate excretion in the urine after each meal[15,16]; while this observation suggests the alkaline tide, Kuyper and Mattill[15] pointed out that eating eggs and toast produced greater citrate excretion than sodium bicarbonate did, although the urine was more alkaline with the latter. In some dogs a high sucrose diet produced greater citrate excretion than a high

TABLE 1.—*Daily urinary excretion of citric acid in man*

AUTHORS	AGE AND STATE	AMOUNT EXCRETED
		mg.
1. Smith, Barnes, et al.	8 infants	64–237
2. McClure and Sauer	39 children	30–431
3. Amberg and McClure	3 adults	440–475
4. Östberg	300 adults	140–1340
5. Sherman, Mendel and Smith	7 adults	356–1180
6. Kissin and Locks	16 normal adults	180–1260
7. Kissin and Locks	16 adults; urolithiasis	30–342

casein diet[16]; combination of a high sucrose diet with alkali caused large excretion of citrate.

4. *Citrate administration.* Ingestion of citrate usually causes an increased urinary elimination of this substance although the yield is not great. The largest increase of urinary citric acid after ingestion of 40 gm. of this material was 1 gm.[13] Kuyper and Mattill[15] found that 1.5–2.5 per cent of ingested citric acid escaped oxidation. Sherman, Mendel and Smith[17] observed in the dog following the feeding of large amounts of citric acid that about 0.7 per cent of the acid appeared in the urine. When fed, successively, chemically equivalent amounts of sodium bicarbonate and sodium citrate, dogs always excreted more citrate on the latter compound[16]. Feeding ammonium citrate to man increased

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[13] Östberg, O.: Skand. Arch. Physiol., **62:** 81, 1931.
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[15] Kuyper, A. C., and Mattill, H. A.: J. Biol. Chem. **103:** 51, 1933.
[16] Sherman, C. C., Mendel, L. B., and Smith, A. H.: J. Biol. Chem., **113:** 247, 1936.
[17] Sherman, C. C., Mendel, L. B., and Smith, M. H.: J. Biol. Chem., **113:** 265, 1936.

the citric acid output threefold[18]. Citric acid fed to infants[12] did not cause a large increase in the urinary excretion of citrate.

5. *Sex hormones.* Shorr and colleagues[18] observed a characteristic cyclic alteration in citrate excretion during different phases of the menstrual cycle, viz. low excretion levels during menstruation with high values about the middle of the cycle. They administered estradiol benzoate to two amenorrheic girls, causing significant increases during treatment with a sharp return to lower levels on discontinuing it; a male with pituitary hypogonadism, during testosterone injection, had a decreased citrate excretion during the treatment with a rise when the hormone was discontinued.

6. *Citrate excretion in urolithiasis.* Boothby and Adams[14] observed that citrate was absent from the urine of two patients with stone. The matter was not further investigated until the important paper of Kissin and Locks appeared; they[19] studied 24 hour specimens of urine of 16 patients known to have calcium stones in the urinary tract, on normal diets, and observed average excretion of 165.3 mg. with ranges from 30–342 mg.; 16 healthy persons, as control, excreted an average amount of 627.6 mg. in 24 hours, with ranges 180–1260 mg. Shorr and co-workers[20] studied 2 men who had a history of recurrent stone formation observing abnormally low amounts of citrate and large amounts of calcium in the urine.

The destruction of citric acid by many types of bacteria has long been known: not all bacteria burn citrate however, since the incubation of citric acid for 44 hours with the feces of infants[12] or of the rat[21] did not destroy this substance. Many tissues, including the kidney, are able to burn citrate. Mårtensson[22] found that venous blood from the kidney was lower in citrate than arterial blood; perfusion of the isolated kidney with citric acid led to rapid disappearance of citrate even when urine is not being secreted. Evidence has been presented[23] that the kidney is able to synthesize citrate from sodium malate.

MATERIALS AND METHODS

Citric acid was determined, colorimetrically, on blood and urine by the method of Pucher, Sherman and Vickery[24] adapted to the Evelyn photoelectric colorimeter; a 4200 Ångstrom filter was used. Ferrous sulphate was employed throughout for decolorizing the oxidation mixture.

Urine was collected for 24 hour periods from 3 men on a normal diet using toluol or concentrated HCl as preservatives. The effects of ammonium chloride, 10 gm. and of citric acid 18 gm. by mouth were studied.

Citric acid, 18 grams, dissolved in water, 250 cc, was given after a breakfast of

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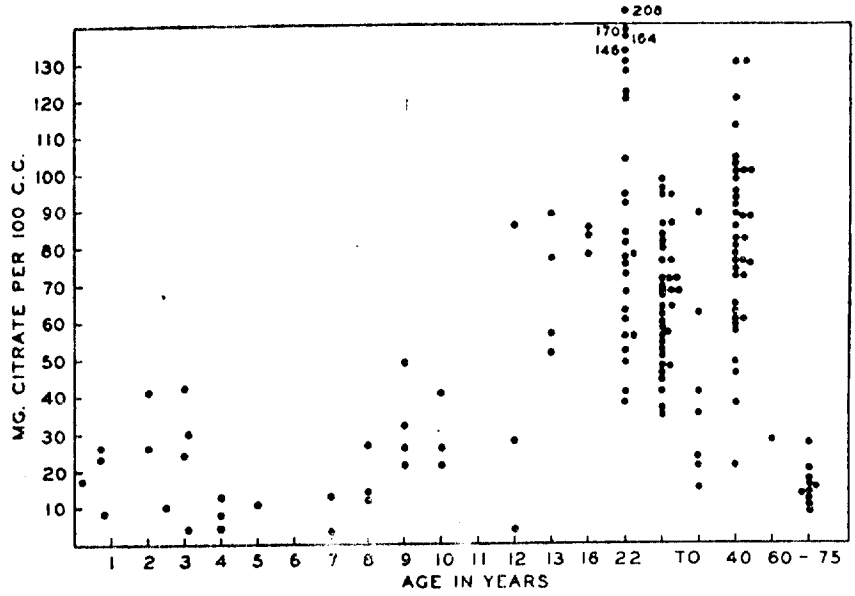

FIG. 1. Citric acid concentration is higher in the urine of young adults than in childhood and old age.

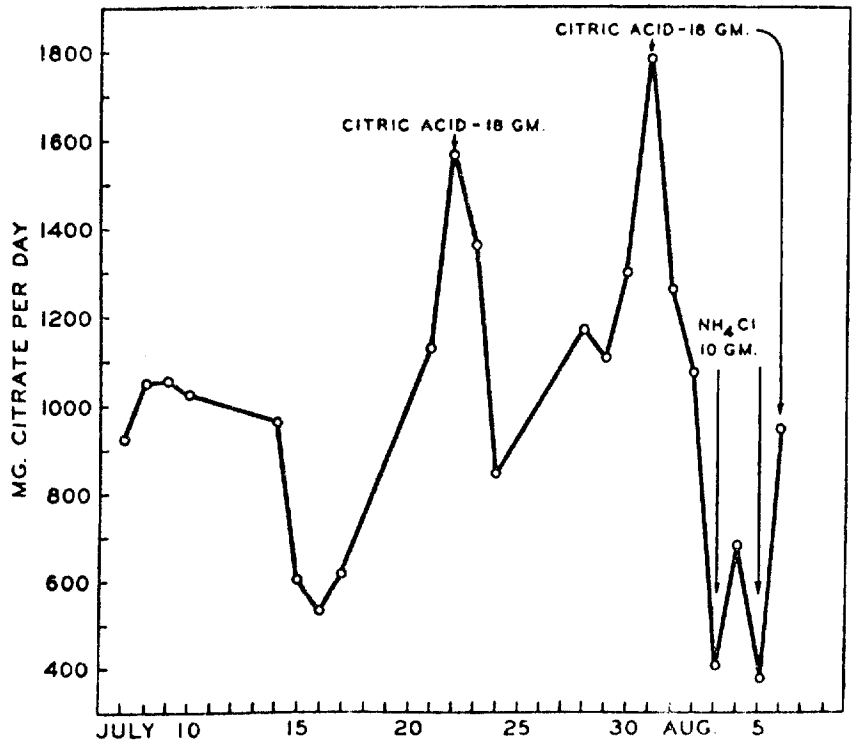

FIG. 2. Daily citric acid excretion of a man aged 40 in good health on a mixed diet; the increase citrate excretion following oral ingestion of citric acid (6 gm. at 4 hour intervals) and the decreased excretion following ammonium chloride, 10 gm. by mouth, are shown.

TABLE 2.—*Citrate excretion following ingestion of citric acid, 18 gm.*

CASE	AGE	FASTING BLOOD CITRIC ACID	HIGHEST BLOOD CITRIC ACID	INITIAL URINARY CITRIC ACID	HIGHEST URINARY CITRIC ACID	TOTAL URINARY CITRIC ACID EXCRETION IN THREE HOURS AFTER INGESTION	UREA CLEARANCES	REMARKS
		Normal persons						
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg.*		
1. D. H.	22	0.9	5.8	120	450	416	65	Normal male
2. C. H.	41	1.5	4.6	88	134	478	46	Normal male
3. W. S.	29	1.5	3.5	80	268	435	72	Normal male
4. T. H.	25	1.3	7.0	50	260	303	48	Normal male
5. D. M.	24	0.3	4.0	110	330	350	60	Normal male
6. J. J.	22	0.9	2.4	50	236	633	44	Normal male
7. B. S.	24	2.0	7.3	30	328	476	48	Normal female
8. B. S.	24	2.2	8.0	50	520	434	50	Normal female
Average		1.32	5.3	72.2	315	441		
		Urolithiasis						
9. J. S.	28	1.2	3.8	0	0	0	67	Recurrent calculus formation. Streptococcus viridans infection
10. P. S.	32	1.3	3.8	30	30	78	54	Multiple unilateral calculi. Uninfected
11. P. S.	32	1.3	5.0	78	105	114	45	Multiple unilateral calculi. Uninfected
12. J. H.	43	1.6	1.9	30	42	59	—	Unilateral nephrolithiasis
13. W. J.	35	1.3	3.2	46	102	116	50	Repeated passage of stones
14. E. K.	31	1.6	6.2	130	168	192	58	Unilateral stones secondary to hydronephrosis
15. J. P.	47	1.8	4.0	20	130	202	44	Bilateral nephrolithiasis
16. C. B.	51	2.2	5.2	37	71	325	35	Unilateral stone. No apparent increase in size for six years
Average		1.5	4.0	47	80	138		

coffee and 1 slice of toast to 16 normal persons and 10 patients who had kidney stones; all of these people were ambulatory. One patient had infection of the

urinary tract with Streptococcus viridans, but the urine of all other patients was free from infection. Blood and urine were obtained just before citrate administration and at ½, 1 and 3 hours after ingestion. The amount of urea in the blood and in the urine excreted in 30 minutes were determined gasometrically and the urea clearance calculated in each instance.

Detailed analysis of the stones was not made. Analysis of 4 stones showed significant amounts of calcium.

RESULTS

The whole blood of 24 young adults contained citric acid 0.9–2.6 mg. in 100 cc, average 1.6 mg. The urine of these persons after a breakfast of toast and coffee contained concentrations of citrate, 26–120 mg. per cent; the average amount was 68 mg. There is less citrate in the urine of children than in adult life and at puberty the concentration approaches that of normal young adults (fig. 1). The urine of 25 children, age 2 months to 11 years, contained citric acid, 4 to 42 mg. per cent, average 21 mg. In 3 old men, 80–84 years, repeated analyses of the urine showed low citrate, values less than 40 mg. per cent being obtained.

There was a fluctuation in the day to day output of citric acid in healthy young males, but no evidence of cycle was obtained as has been described in women[18]. Ammonium chloride, 10 gm., by mouth, in 2 normal men, caused a decrease in the citric acid excretion and citric acid, 18 gm., orally, produced a marked elevation (fig. 2). In addition to the values obtained in old age, there is evidence that citric acid excretion decreased in states of debility. A man with chronic nephritis and uremia during one week excreted less than 150 mg. of citric acid daily. A woman, age 64, and free from calculi, who had a nephrectomy for a solitary renal cyst excreted citric acid, less than 140 mg. daily for 12 days in the postoperative period.

In the presence of calcified stones the urinary citrate excretion is low. The blood citrate in eight of these patients was essentially the same as the normal controls. Following the ingestion of citric acid 18 gm., with 1 exception, the blood citrate rose as high as in the controls; the urinary excretion, however, was considerably decreased (table 2).

DISCUSSION

The increased citrate excretion in young adults, beginning at puberty in both male and female, contrasted with children and old men suggests a hormonal relationship; this suggestion supports the observation of Shorr et al.[18] who have demonstrated that in women a rise of citrate occurs in the middle of the menstrual cycle presumably related to estrogen.

The decreased citrate excretion in calcium nephrolithiasis is in agreement with the discovery of Kissin and Locks[19] which has been confirmed by Shorr[20] and gives support to the metabolic theory of origin of certain calcified stones in the urinary tract. It is obvious that a decreased urinary citrate excretion can represent either diminished alimentary absorption of citrate or unusually effi-

cient mechanisms for burning citrate before reaching the kidney, in the kidney itself or in the urine.[25]

With reference to the last consideration, it has long been known that many, but not all, types of bacteria can burn citric acid; in the present series, only one patient with nephrolithiasis (table 2, case 9) had urinary infection.

Analyses of blood and urines after the ingestion of amounts of citric acid sufficient to elevate the blood citrate should test absorption from the bowel as well as prerenal and renal catabolism of citric acid. The citric acid content of the blood of patients with nephrolithiasis (average, 1.5 mg. per cent) was not significantly different from the normal values (average, 1.32 mg. per cent.). Following administration of citric acid, 18 grams, all patients except one (table 2, case 12) had an appreciable elevation of blood citrate which was only slightly less than normal. From this we deduce that absorption and prerenal catabolism do not differ greatly in nephrolithiasis from in the normal state. However, there is a greatly diminished excretion of citric acid in most of the patients with stone, even in the absence of infection. Presumably the oxidation of citric acid occurs in the kidney although there is no rigorous proof except in infections that it does not occur in the urine.

Following oral administration of 0.5–2.0 gm. of citric acid per kilo to dogs, Sherman, Mendel and Smith[17] observed an average urinary excretion of 0.7 per cent of the acid; the rise in blood citrate was maintained for $3\frac{1}{2}$ to $7\frac{1}{2}$ hours.

The mechanism of excretion of citrate by the kidney is uncertain. Calculations of citrate clearances from the data available from the tolerance studies, indicate that citrate clearances roughly parallel simultaneous urea clearances. This evidence, together with the type of curve developed by plotting plasma concentration of citrate against total citrate excretion suggests a filtration and a resorptive mechanism for citrate. However, any theoretical consideration is hindered by the fact that all citrate determinations were made on venous, not

[25] Citrate may also be decreased in urine by entering the solid phase of urinary stones, of which it is a frequent constituent. In 100 such calculi analyzed, only 7 contained no citric acid. The following concentrations of citrate were found, the highest value being 3.34%;

0.01	0.1%,	18 stones
0.1	1.0%,	59 stones
1.0	2.0%,	8 stones
2.0	3.0%,	4 stones
3.0	3.34%,	2 stones

Detailed analyses were made of 9 stones containing the largest amounts of citric acid, 1.6–3.34%; the results expressed with reference to dry weight, follow:

	mg. per 100 mg.
Calcium	25.5-37.5
Magnesium	3.0- 5.7
Phosphorus	7.8-19.75
Oxalate	6.9-52.8

The solubility of calcium citrate, $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$, has been reported to be 2.5 gm. per liter (about 0.004 Molar) at 30° (Archiv. Pharm., **241:** 413, 1930). Adsorption or the formation of a complex salt might cause citrate to appear in stones, even if the solubility of calcium citrate as such is not exceeded.

arterial bloods. A priori, lower citrate values for venous blood from the kidney are obtained than for arterial blood

The results show that usually it is not possible to significantly increase urinary citrate in the urine of stone patients with citric acid by oral administration of a single large dose of this substance. It is apparent that excretion of the large amounts of citrate which occur in the urine following citric acid by mouth (as much as 1.9 gm. daily in our experience) fall in the range where effect in decalcification of stones might take place; what is needed clinically are methods to significantly increase citrate excretion in an acid urine in patients with stone.

CONCLUSIONS

Compared with children and debilitated persons, aged men, increased amounts of citric acid are excreted in the urine of normal adults beginning at puberty.

In the presence of calcified urinary calculi the urinary citrate is lower in concentration than in normal adults while the blood citrate is at a normal level. Feeding large amounts of citric acid to patients with urolithiasis increases the blood citrate as in the normal but much less citrate is excreted in the urine than occurs in the controls. Absorption from the bowel and prerenal catabolism of citrate in urolithiasis are not abnormal, but more efficient mechanisms exist in stone patients for oxidation of citrate in the kidney or urine than in healthy persons.

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THE METABOLISM OF CITRIC ACID BY INFANTS[1]

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It has long been known that the salts of the organic acids occurring in fruits and vegetables are, with few exceptions, metabolized to alkaline end-products in the body. The extensive literature on this point has been reviewed by Smith and Orten ('37). Furthermore, the observation has been made that, like natural fruit juices, the ingestion of sodium citrate results in an increased pH of the urine and an augmented excretion of citric acid whereas an equivalent amount of free citric acid has little obvious influence on the composition of the urine (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34). It has been shown that the alkalinization produced by sodium bicarbonate and by sodium acetate likewise increases the citric acid in the urine from which evidence the conclusion has been reached that the organism has the ability to synthesize citric acid (Schuck, '34a,b; Sherman, Mendel and Smith, '36a).

The distinct metabolism of free citric acid has been emphasized by the almost complete disappearance (insofar as examination of the urine is concerned) of this compound in adult human subjects (Östberg, '31; Kuyper and Mattill, '33; Schuck, '34b), in the dog (Sherman, Mendel and Smith, '36b), in the pig (Woods, '27; v. Fürth, Minnebeck and Edel, '34)

[1] A preliminary report was presented before the American Institute of Nutrition, New Orleans, March, 1940.

[2] Chas. Pfizer and Co. Research Fellow in Physiological Chemistry, 1938–39.

and in the rat (Kuether, Meyer and Smith, '40) after administration by mouth. Gonce and Templeton ('30) examined the urine of four children ranging from 7 to 12 years of age before and after giving 4 gm. of "dehydrated" citric acid per 15 pounds body weight. The results were irregular but in one subject the ingestion of the free acid resulted in a pronounced drop in the concentration of citric acid in the urine. In a study of the factors influencing the retention of calcium, phosphorus and nitrogen of infants who were fed whole milk, Jeans and co-workers ('36) made a comparison of the metabolism of the food citrate on the one hand, and of added citric acid, on the other. With four infants ranging in age from 7 weeks to 26 weeks, 0.088 to 0.206 gm. of citric acid were excreted in the urine when 1.47 to 2.72 gm. per day of food citrate were consumed; when, after adding free citric acid as a curdling agent to the milk, the citric acid intake was increased from 3.16 to 5.02 gm., the output remained at 0.097 to 0.213 gm. These data indicate complete destruction of citric acid when fed as such to this age group.

In their study of the metabolism of orally administered citric acid in the dog, Sherman, Mendel and Smith ('36b) found no increase in fecal citric acid when from 0.5 to 2.0 gm. of the free acid per kilo body weight was given. Langecker ('33) detected no citric acid in the feces of rabbits after feeding either sodium citrate or citric acid. The studies of Kuether, Meyer and Smith ('40) show that the feces of rats contain small amounts of citric acid and that the administration of free citric acid has little, if any, influence on the quantity lost by way of the intestine.

That considerable amounts of citric acid appear in the feces of human subjects was demonstrated by Smith, Bauguess and Barnes ('39) in a study of eleven infants from 3 to 14 months old; they reported analyses showing that from 1.0 to 7.8% of the citrate ingested by this group appeared in the feces. The present study is an extension of their work; it deals with the comparison of the metabolism of food citrate and of free citric acid as determined by the balance method.

EXPERIMENTAL PART

Methods

The subjects of this study were eight infants from 4 to 12 months of age with varying degrees of mild clinical rickets but without other abnormalities. They were given as much as they would voluntarily consume of a formula made up of dried milk, barley flour and *B*-lactose and also canned cereal, soup and apricot-apple sauce. Accurate account of food consumption was kept at all times. During the collection periods (3 days in length and marked by carmine) the babies were maintained on metabolism frames which permitted a complete separation of urine from feces and accurate quantitative collection of both. The data discussed herein are derived from one period during which free citric acid (365 mg./kg. body weight per day), was given by gavage in divided doses and one or two basal periods without added citric acid, for each subject.

The urine was collected in hydrochloric acid and the feces were digested with concentrated hydrochloric acid to a uniform suspension. For the determination of citric acid in the food, feces and urine, the method of Pucher, Sherman and Vickery ('36) was used, the final measurement being made photoelectrically, using a color filter having a maximum transmission at 4250 Å. Approximately 1 gm. samples of the separate food materials were thoroughly stirred with acid (70 cc. water and 3 cc. concentrated sulfuric acid) and extracted on a hot plate; after making to volume and filtering, the determination was made directly on the filtrate in most cases. The reconstituted dried milk was treated with trichloracetic acid and the determination made on the protein-free filtrate. The analyses for the citrate content of the food materials appear in table 1. Inasmuch as it was shown that all of the citric acid of the digested fecal suspension was in the fluid part, samples for analysis were measured directly with a pipette.

DISCUSSION OF RESULTS

It is obvious from the analyses of the food materials shown in table 1 that all of the constituents of the diet contained citric acid but that the dried milk and the canned fruit mixture were particularly rich in this respect. In the course of the experiment the food of the individual infants contributed from 1.091 to 2.502 gm. citric acid per day (see table 2).

In the report of Smith, Bauguess and Barnes ('39) attention was called to the not inconsiderable elimination of citric acid in the feces although it was pointed out that the larger part of the excretion was through the urine. In the present study it is shown again that the feces always contain citric acid in varying proportions of the intake but ordinarily less than that appearing in the urine. The source of this citric acid may be the food, digestive or other body fluids entering the intestine or the activity of the intestinal bacteria. Incubation of known amounts of citric acid with infant feces for 44 hours produced, in our experience, neither destruction nor formation of citric acid. This observation agrees with that of Langecker ('34) who found that the bacteria and enzymes

Table I.
Citric Acid Content of Diet Constituents

Material	Citric Acid
	Per Cent
Barley Flour	0.047
* Milk Powder	1.400-1.625
B-Lactose	0.000-0.006
** Cereal	0.132
** Soup	0.078
** Apricot and Apple Sauce	0.638

* Klim
** Gerber's

in the intestinal tract of the rabbit do not destroy citric acid. In another study (Kuether, Meyer and Smith, '40) it has been shown that incubation of the intestinal contents of the rat with free citric acid for 44 hours results in no detectable destruction or formation of citric acid. In subjects ER, TA, WF and MC the large losses of citric acid in the feces during the period of free citric acid administration are correlated with diarrhea, probably induced by the citric acid itself. The food and possibly the intestinal secretions, thus appear to be responsible for the presence of citric acid in the feces. It is worthy of comment that, in the absence of diarrhea, fecal citric

Table II. CITRIC ACID BALANCE

Subject	Body Wgt	Citric Acid Intake per 24 hrs			Citric Acid Excretion per 24 hrs			Excretion of Citric Acid in Per Cent of Total Intake			Citric Acid Absorbed in Per Cent of Total Intake	Urine Absorbed X 100	Urine Intake X 100
		Food	Extra	Total	Urine	Feces	Total	Urine	Feces	Total			
	KG	GM	GM	GM	GM	GM	GM						
ER ♂	4.94	1.694		1.694	.168	.126	.294	9.9	7.4	17.3	92.6	10.7	9.9
	5.93	1.174	2.00	3.174	.095	.416	.511	3.0	13.1	16.1	87.0	3.7	8.1
A.K ♂	8.32	1.909		1.909	.161	.068	.229	8.4	3.6	12.0	96.4	8.7	8.4
	8.76	2.502		2.502	.165	.079	.244	6.7	3.2	9.9	96.8	6.9	6.6
	8.19	1.091	3.00	4.091	.094	.032	.126	2.3	0.8	3.1	99.2	2.3	8.6
F.F ♀	8.23	1.325		1.325	.108	.093	.201	8.1	7.0	15.1	93.0	10.4	9.6
	7.81	1.995	2.70	4.695	.062	.086	.148	1.3	1.8	3.1	98.0	1.3	3.1
T.A ♂	10.34	2.464		2.464	.231	.079	.310	9.6	3.0	12.6	97.0	9.9	9.6
	9.64	2.149	3.30	5.449	.101	.65	.751	1.8	11.9	13.7	86.0	2.1	4.7
J.A ♂	8.46	2.007		2.007	.230	.046	.276	11.5	3.5	15.0	96.5	11.9	11.4
	7.25	1.654	2.59	4.834	.130	.080	.240	3.8	1.9	5.7	98.0	3.9	9.7
R.P ♂	7.03	1.328		1.328	.087	.067	.154	6.6	5.0	11.6	95.0	7.0	6.6
	8.05	1.788		1.788	.132	.173	.305	7.4	9.7	17.1	90.3	8.2	7.4
	6.67	1.972	2.58	4.372	.042	.340	.382	0.9	8.0	8.9	97.0	1.0	2.5
W.F ♂	9.43	2.061		2.061	.249	.040	.308	12.9	1.9	14.8	98.1	13.1	12.9
	10.57	2.707		2.707	.221	.073	.294	10.0	3.3	13.3	96.7	10.3	10.0
	9.30	1.954	3.18	5.134	.072	1.000	1.072	1.4	19.5	20.9	80.0	1.8	3.7
M.C ♂	8.25	1.760		1.760	.142	.078	.220	9.2	3.3	12.5	94.7	9.5	9.2
	7.68	1.628	2.78	4.408	.064	1.066	1.130	1.5	24.1	25.6	76.0	2.0	3.9

acid represents a lower proportion of ingested citric acid in the periods when the free acid is fed than in the control periods.

The above observations permit the conclusion that the citric acid which disappears from the intestinal tract is absorbed. From table 2 it can be seen that the proportion of ingested citric acid which is absorbed is high but is decreased in those instances where diarrhea was present. A different picture is presented by the data for citric acid excretion in the urine; here both absolutely and in relation to the quantity ingested, the values for the concentration of citric acid are strikingly

lower during the period when free citric acid was fed than in the control periods when the citric acid was obtained only from the food. A comparison of the proportion of ingested citric acid which was absorbed with the proportion appearing in the urine, provides an index of the completeness of metabolism of this substance. The thirteenth column in table 2 indicates that within the control periods and within the free citric acid-feeding periods, the extent of metabolism of the absorbed citric acid is uniform for the various subjects. However, when one compares the extent of metabolism of the free acid period with that of the control periods, there is a striking difference; the proportion of the absorbed citric acid escaping destruction averaged 9.6 (6.9 to 13.1) % in the control periods and 2.3 (1.0 to 3.9) % in the free acid periods. In the light of the failure to store citric acid in the body under ordinary conditions (Smith and Orten, '37) these data strongly suggest the interpretation that ingested free citric acid is either largely destroyed in the body or transformed to a non-citrate type of compound (see MacKay, Carne and Wick, '40).

A comparison of the amount of citrate ingested in the food with the concentration of urinary citric acid may be taken as an index of the influence of the diet on endogenous citric acid production inasmuch as both the salts of the organic acids occurring in foods and the major foodstuffs themselves, are known to affect citric acid synthesis. Such a comparison, shown in table 2, indicates that in seven of the eight subjects, the ingestion of free citric acid decreases the ratio of urinary to food citric acid, which fact may be interpreted to indicate that not only is the free citric acid metabolized but that, under its influence, the basal production of citric acid as affected by the diet, is inhibited. A similar comparison of the data from like experiments with the rat (Kuether, Meyer and Smith, '40), shows that, whereas the administered free citric acid completely disappears with this species it does not interfere to the same extent with the formation of urinary citric acid as influenced by the diet. The synthesis of citric acid, which can be demonstrated in the rat (Smith and Meyer, '39, Kuether,

Meyer and Smith, '40) is not so striking in the human subjects herein described.

Experimental data are available showing that under certain conditions, the excretion of citric acid by the kidney is suppressed. Thus the administration of hydrochloric acid or of potentially acid substances like calcium chloride and ammonium chloride, is followed by a decrease in citrate concentration in the urine (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34). Again, Smith and Meyer ('39) have demonstrated that in the rat, the production of endogenous citric acid is greatly diminished when the animal is maintained on a high-protein diet. In the present investigation the failure to increase urinary citric acid apparently is not due to its effect on the acid-base equilibrium for it has been shown by Schuck ('34b) that ingestion of the free acid produces little if any variation of the pH of adult human urine and the same observation has been made in the rat (Kuether, Meyer and Smith, '40).

The present study shows again that the metabolism of free citric acid is not the same as that of food citrate or sodium citrate insofar as can be judged by the excretion of citric acid in the urine. That the metabolism of the free acid exerts a marked effect on that of food citrate is demonstrated by the data. From these balance experiments one can conclude that the fate of food citrates and of free citric acid in the infant is like that in adults and in the other mammals which have thus far been studied.

SUMMARY

Based on the data derived from a series of balance studies with infants, it has been shown that the oral administration of free citric acid results in a marked decrease in the proportion of the ingested citric acid and food citrate appearing in the urine, as compared with that of the control period in which the basal diet is the only source of citric acid. Ordinarily, the feces contain a small amount of citric acid; the citric acid of intestinal contents is uninfluenced by the addition of free citric acid.

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RENAL CITRATE CLEARANCE IN INFANCY

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To check the participation of the kidneys in the metabolism of citrate in man the clearance technique was used in 1964 (1). We were unable to determine in available literature data on the use of the clearance technique in checking the participation of the kidneys in the metabolism of citrate in infancy. Partial data from this age period are mostly incomplete and pertain only to some indicators of renal participation in the metabolism of citrate. Under these conditions it is difficult to consistently use the findings from the physiology and pathology of adults in diagnosis and perhaps also in the therapy of disturbances of the metabolism of citric acid in infancy. Disturbances in the metabolism of citric acid were already noted in many pathological conditions. These are mainly various endocrinopathies, diseases of the bone system, malignant diseases of the kidneys, liver, heart, congenital metabolic diseases and disturbances in nourishment. Many drugs and metabolically-active substances, including vitamins, also bring about changes in the metabolism of citric acid.

In view of the above we decided to investigate some parameters in infants of the participation of the kidneys in the metabolism of citrate, this being done both in twenty-four hour samples of urine and in short-term observations.

Materials and Method

We investigated clearance of citrate in twenty-four hour samples of urine in 116 healthy eutrophic infants placed in the Infant Institute in Košice. We checked only male infants (because of the easier possibility of collecting urine in quantity). The infants were on a set formula regime for at least two to three weeks. Before the investigation they did not receive vitamin D. We took blood for checking the plasmatic concentration of citrate in the morning on an empty stomach in test tubes with dried heparin. After taking the blood after spontaneous urination we began to catch the twenty-four hour uncauterized urine, the collection of which also ended with spontaneous urination. The beginning and end of the collection of urine was conducted under the supervision of a doctor. We collected the urine in bottles into which we placed 0.5 ml of 10% thymol in absolute alcohol as a conserving reagent.

We made the short-term investigation of the clearance of citrate in 19 healthy eutrophic infants of both sexes. This was done by the standard clearance technique with the use of an infusion of inulin. We did the checking in the morning after night fasting. In selecting the infants we used similar criteria as in infants with the twenty-four collection of urine.

We observed the maximal tubular resorption of citrate (Tm) after an intravenous application of a starting dose and a maintaining dose of 3% citrous acid, that is, natriumcitrate, in a physiological solution. We gave the starting dose in the quantity of about 0.2m Mol/1 kg, the maintaining dose in the quantity of about 0.01m Mol/1 kg/min.

We determined citrate both in the plasma and in the urine according to Gey, inulin by the modified method according to Vesterdal, creatinine according to Momos (for details see 8).

sults

The average values of citrate clearance from the twenty-four hour samples of urine fluctuated in individual months of life between 5.37-8.01 ml/min./1.73 m^2 of body surface with a tendency toward higher values in the first months of life. The average value of citrate clearance (with standard deviation) for the entire period of infancy was 6.17±2.58 ml/min./1.73 m^2 of body surface.

In the short-term investigations with the use of the standard clearance technique we verified the average value of citrate clearance 7.78±3.68 ml/min./ 1.73 m^2 of body surface. Tubular resorption of citrate expressed in absolute values was 0.026±0.007 mg/l ml of glomerular filtrate, expressed in relative values 91.91 ± 4.47% from the value of the filtrated quantity. Individual values of the listed parameters, including clearance of inulin are in the table.

With an increase of citratemia (by the parenteral induction of citrous acid, that is, natriumcitrate) in the average 5.4 times as against the control value we did not determine in infants the existence of maximal tubular resorption of citrate.

Discussion

The quantity of plasma cleared with some substance for a unit of time during the passage of blood through the kidneys depends on the prefiltrated quantity and on the processes arrived at during the passage of the primary urine through the tubular apparatus. In the citrate, the quantity prefiltered during a unit of time is calculated as a product of glomerular filtration and concentration of citrate in the plasma since thus far the value of Donnan's factor for this substance is not known.

The quantity of glomerularly filtered citrate is not under normal conditions the determining factor of the value of citrate clearance (1). Under certain

conditions however the quantity of prefiltrated citrate can become a determining factor of its excretion. Thus Grollman et al.(4) established that with the infusion of l-malate (but also other intermediates of the Krebs cycle) in dogs the ratio of $\frac{C\ citrate}{C\ exog.creat.}$ increases quickly and this to such a degree that citrate clearance can reach the value of the clearance of exogennic creatinine. Under these conditions then the prefiltrated citrate will quantitatively be excluded by the urine. Also with metabolic alkalosis the value of the citrate clearance can increase even to the value of glomerular filtration (4) as a result of the hampering of oxidation of the resorbed citrate in the mitochondria of the tubular cells.

We evaluated the participation of the tubular apparatus at excretion of substances by the variance in the glomerularly filtrated and released by the urine quantity of a given substance. The standard clearance technique therefore expresses only the summary action and does not catch all the processes, or the bidirectional movement of substances, as experimental methods allow, for example, the micropunct and "stop flow" method. With the aid of these it was shown that in the evaluation of the participation of the tubular apparatus in the metabolism especially of organic substances, one must reckon with a combination of processes or resorption, secretion, diffusion, synthesis, utilization and bilateral non-ionic diffusion (3).

Under physiological conditions, with the participation of the kidneys tubular functions, especially resorption, are a decisive factor in the metabolism of citric acid. The majority of authors take the view that citrate is not excreted by the tubules (1,4). The question of tubular secretion of citrate however is not yet fully resolved because secretion of other intermediates of the Krebs cycle has already been verified also in chicks in which the tubular circulation is separated from the glomerular

by the existence of the vein portae renalis (5). The bidirectional tubular movement of malate was also verified by the "stop flow" method.

Several authors verified both the synthesis and the utilization of citrate in the kidneys. Moreover the tubular cells do not utilize only the citrate from the peritubular blood but also from the tubular fluid (4). We verified the synthesis and utilization of citrate in vivo (in dogs) also in our own research (8) and we observed that the transition of one process to the other and vice versa can take place in a relatively short time. Whether citric acid in the kidneys is utilized or produced predominantly is probably determined mainly by the functional condition of the tubular apparatus and the concentration in the blood of the utilized substrata in the blood (3). Changes in the acidobasal balance (8) which are also the main factor influencing the non-ionic diffusion of substances through the cellular membranes (3) have a great influence on the metabolism of citrous acid in the cellular tissue.

The clearance of citrate is the resulting value of the action of the listed tubular processes which can be in the quantitative and the qualitative sense considerably influenced both under physiological conditions and also by the action of various substances and under various pathological conditions. Moreover the greatest significance is attributed to hormones, disorders in the acidobasal balance, vitamin D, dietetic intervention, intermediates of the Krebs cycle, nephropathies, and to several chemicals (5). According to Rechnenberg et al. (7) an important role is also played by the age of the individual.

Shorr et al. (cit. acc. to 1) determined in adults in a twenty-four hour sample of urine clearance of citrate about 20 ml/min., which agrees basically also with findings in the short-term investigations of Canary et

et al.(1) and this was 17 ml/min./100 ml of glomerular filtrates (bordering on 8-22 ml). According to these authors tubular resorption represents the value of about 85% filtrated quantity of citrate (with the use of clearance of endogennic creatinine as gauges of glomerular filtration).

The values of citrate clearance which we verified in infants in the twenty-four hour samples, and also in the short-term collections of urine, represent barely a third to half of the listed values of adults. This difference is limited mainly by the considerably higher tubular resorption of citrate in infancy (almost 92%). The difference in contrast to the data in the literature (1) results however also from the diverse method of measuring glomerular filtration. After recalculating to 100 ml of glomerular filtration, measured with the aid of clearance of endogennic creatinine, the value verified by us represented about 63% of the value which is given by Canary et al. (1) in contrast to 46% with the use of inulin as a measure of glomerular filtration. Both as in adults and in infants however endogennic creatinine is not very suitable for the accurate measurement of the value of glomerular filtration. The main essence of the difference between our results with the data in the literature (1) probably rests on the peculiarities of the metabolism in infancy in general and in the kidneys in particular.

The differences between the values of citrate clearance which we obtained from the twenty-four hour samples of urine and from the short-term collections, can be qualified by several factors. An important role can be played by the time of the collection of the urine because even when a quantity of citrate excreted during twenty-four hours is relatively constant during the course of a day it varies relatively considerably. Also important is the application of various factors which vary relatively considerably during the collection of

the twenty-four hour samples of urine and during the use of the clearance technique (1). In the twenty-four hour collection of urine the influence of nourishment received is obviously valid and this not only directly but also by its physiological consequences in the area of acidobasal balance and in the composition of its internal surroundings (5).

After increasing the plasmatic concentration of citrate on the average to 13.64 mg% we did not determine the existence of maximal tubular resorption of citrate. Grollman et al.(4) also did not determine (in experiments on dogs) the existence of maximal tubular resorption of citrate, and this not even with an increase of plasmatic concentration of citrate on toxic values.

Investigation of the participation of the kidneys on the metabolism of citrate in infancy can be one of the ways of early determination of metabolic malfunctions in general and in particular kidney parenchyma. Besides total disorders in the action of the kidneys they can aid in uncovering disorders in the regulation of acidobasal balance because the excretion of citrate also represents a defensive mechanism with the aid of which the kidneys can conserve fixed anionts. This defensive mechanism was already ascertained also in the newly-born infants (6).

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Table 1. Values of inulin clearance (C_{in}), clearance (C_{Ci}) and tubular resorption of citrate (R_{Ci}) in infants

Comparison number	Age in months	C_{in}	C_{Ci}	R_{Ci} v %
		ml/mm/1,73 m²		
1	1	87,54	5,01	94,28
2	4	96,06	4,91	94,89
3	5	92,28	5,16	94,41
4	5	99,04	13,55	86,32
5	6	80,60	13,80	82,87
6	6	88,47	3,66	95,86
7	7	118,40	6,11	94,84
8	7	111,46	8,08	92,79
9	8	105,18	6,43	93,89
10	9	71,58	7,10	90,09
11	9	69,02	13,09	81,03
12	9	89,78	4,57	94,91
13	10	148,71	12,95	91,29
14	10	94,26	3,40	96,40
15	10	115,08	9,92	91,38
16	12	93,46	3,28	96,50
17	12	82,74	7,43	90,17
18	12	112,27	12,26	89,08
19	12	150,46	7,10	95,28
Average	8	100,33	7,78	91,91
Adults (1)			20,00	85,00

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OBLIČKOVÁ CLEARANCE CITRÁTU V DOJČENSKOM VEKU

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Na sledovanie účasti obličiek na metabolizme citrátu u ľudí bola použitá clearanceová technika v roku 1964 (1). V dostupnej literatúre sa nám nepodarilo zistiť údaje o použití clearanceovej techniky pri sledovaní účasti obličiek na metabolizme citrátu v dojčenskom veku. Dielčie údaje z tohto vekového obdobia sú zväčša kazuistického charakteru a týkajú sa len niektorých ukazovateľov obličkovej účasti na metabolizme citrátu. Za týchto podmienok je ťažko dôsledne využívať poznatky z fyziológie a patológie dospelých ľudí v diagnostike a snáď aj v terapii porúch metabolizmu kyseliny citrónovej v dojčenskom veku. Poruchy metabolizmu kyseliny citrónovej boli už zaznamenané u mnohých chorobných stavov. Hlavne sú to rôzne endokrinopatie, ochorenia kostného systému, maligne ochorenia, choroby obličiek, pečene, srdca, vrodené metabolické ochorenia a poruchy výživy. K zmenám metabolizmu kyseliny citrónovej dochádza tiež po mnohých farmakách a metabolicky účinných látkach, včítane vitamínov.

Vzhľadom na uvedené rozhodli sme sa sledovať u dojčiat niektoré parametre účasti obličiek na metabolizme citrátu, a to ako v 24-hod. vzorkách moča, tak aj v krátkodobých sledovaniach.

Materiál a metóda

Clearance citrátu z 24-hod. vzoriek moča sme vyšetrovali u 116 zdravých eutrofických dojčiat, umiestnených v Dojčenskom ústave v Košiciach. Vyšetrovali sme len dojčatá mužského pohlavia (pre ľahšiu možnosť kvantitatívneho odberu moča). Dojčatá boli na ustálenom režime umelej výživy aspoň 2—3 týždne, pred vyšetrovaním nedostali D vitamín. Krv na vyšetrenie plazmatickej koncentrácie citrátu sme odoberali ráno na lačno do skúmaviek s vysušeným heparínom. Po odobratí krvi sme po spontánnom vymočení začali zachytávať 24-hod. nekatetrizovaný moč, ktorého odber sa tiež skončil spontánnym vymočením. Počiatok aj ukončenie odberu moča sa dialo pod kontrolou lekára. Moč sme zachytávali do fliaš, do ktorých sme ako konzervačné činidlo dávali 0,5 ml 10% tymolu v absolútnom alkohole.

Krátkodobé vyšetrenie clearance citrátu sme uskutočnili u 19 zdravých eutrofických dojčiat obojeho pohlavia, a to štandardnou clearanceovou technikou za použitia infúzie inulínu. Vyšetrenia sme robili ráno po nočnej hladovej prestávke. Pri výbere dojčiat sme použili rovnaké kritéria ako u dojčiat s 24-hod. odberom moča.

Maximálnu tubulárnu reabsorpciu citrátu (Tm) sme sledovali po vnútrožilovom podaní nárazovej a udržiavacej dávky 3% kyseliny citrónovej, resp. nátriumcitrátu vo fyziologickom roztoku. Nárazovú dávku sme podávali v množstve asi 0,2 m Mol/1 kg, udržiavaciu dávku v množstve asi 0,01 m Mol/1 kg/min.

V plazme aj v moči sme stanovovali citrát podľa Geya, inulín modifikovanou metódou podľa Vesterdala, kreatinín podľa Momosa (podrobnejšie pozri 8).

Výsledky

Priemerné hodnoty clearance citrátu z 24-hod. vzoriek moča sa pohybovali v jednotlivých mesiacoch života v rozmedzí 5,37 –8,01 ml min./1,73 m^2 telesného povrchu s tendenciou k vyšším hodnotám v prvých mesiacoch života. Priemerná hodnota clearance citrátu (so štandardnou deviáciou) pre celý dojčenský vek bola 6,17 ± 2,58 ml/min./1,73 m^2 telesného povrchu.

Pri krátkodobých sledovaniach za použitia štandardnej clearanceovej techniky sme zistili priemernú hodnotu clearance citrátu 7,78 ± 3,68 ml/min./1,73 m^2 telesného povrchu. Tubulárna rezorpcia citrátu, vyjadrená v absolútnych hodnotách bola 0,026 ± 0,007 mg/1 ml glomerulárneho filtrátu, vyjadrená v hodnotách relatívnych 91,91 ± 4,47 % z hodnoty filtrovaného množstva. Individuálne hodnoty uvedených parametrov, včítane clearance inulínu, sú na tabuľke.

Pri zvýšení citratémie (parenterálnym prívodom kyseliny citrónovej, resp. nátriumcitrátu) v priemere 5,4-krát oproti kontrolnej hodnote nezistili sme u dojčiat existenciu maximálnej tubulárnej rezorpcie citrátu.

Diskusia

Množstvo plazmy, očistené o nejakú látku za jednotku času, pri prietoku krvi obličkami závisí od prefiltrovaného množstva a od procesov, ku ktorým dochádza pri prietoku primárneho moča cez tubulárny aparát. U citrátu sa množstvo prefiltrované za jednotku času vypočítava ako súčin glomerulárnej filtrácie a koncentrácie citrátu v plazme, keďže zatiaľ nie je známa hodnota Donnanovho faktoru pre túto látku.

Množstvo glomerulárne filtrovaného citrátu nie je za normálnych podmienok určujúcim faktorom hodnoty clearance citrátu (1). Za určitých podmienok sa však množstvo prefiltrovaného citrátu môže stať rozhodujúcim faktorom jeho exkrécie. Tak Grollman a spol. (4) zistili, že pri infúzii l-malátu (ale aj iných intermediátov Krebsovho cyklu) u psov rýchle stúpa pomer $\frac{C_{citrátu}}{C_{exog.\ kreat.}}$ a to až tak, že clearance citrátu môže dosiahnuť hodnotu clearance exogénneho kreatinínu. Za týchto pomerov sa teda prefiltrovaný citrát kvantitatívne vylúči močom. Tiež pri metabolickej alkalóze môže stúpať hodnota clearance citrátu až na hodnotu glomerulárnej filtrácie (4) v dôsledku brzdenia oxidácie rezorbovaného citrátu v mitochondriách tubulárnych buniek.

Účasť tubulárneho aparátu pri exkrécii látok hodnotíme z rozdielu glomerulárne filtrovaného a močom vylúčeného množstva danej látky. Štandardná clearanceová technika vyjadruje teda len sumárne deje a nezachytáva všetky procesy, prípadne biderekcionálny pohyb látok, ako to dovoľujú experimentálne metódy, napr. mikropunkčná a „stop flow" metóda. Pomocou týchto sa ukázalo, že pri hodnotení účasti tubulárneho aparátu na metabolizme, zvlášť organických látok, treba počítať s kombináciou procesov rezorpcie, sekrécie, difúzie, syntézy, utilizácie a obojstrannej neionickej difúzie (3).

Za fyziologických podmienok sú pri účasti obličiek na metabolizme kyseliny citrónovej rozhodujúce tubulárne funkcie, hlavne rezorpcia. Väčšina autorov zastáva názor, že citrát sa tubulami nesecernuje (1, 4). Otázka tubulárnej sekrécie citrátu však ešte nie je plne doriešená, pretože už bola dokázaná sekrécia iných intermediátov Krebsovho cyklu a to aj u kurčiat, u ktorých je tubulárna cirkulácia oddelená od glomerulárnej existenciou vena portae renalis (5). Tiež „stop flow" metódou bol dokázaný bidirekcionálny tubulárny pohyb malátu.

Viacerí autori dokázali tak syntézu, ako aj utilizáciu citrátu v obličkách. Tubulárne bunky pritom neutilizujú len citrát z peritubulárnej krvi, ale aj z tubulárnej tekutiny (4). Syntézu aj utilizáciu citrátu in vivo (u psov) sme zistili

aj pri vlastných sledovaniach [8] a pozorovali sme, že prechod jedného procesu v druhý a naopak môže sa diať v relatívne krátkom čase. O tom, či je kyselina citrónová v obličkách prevažne utilizovaná alebo produkovaná, rozhoduje asi hlavne funkčný stav tubulárneho aparátu a koncentrácia utilizovatelných substrátov v krvi [3]. Veľký vplyv na metabolizmus kyseliny citrónovej v tkanive majú zmeny acidobázickej rovnováhy [8], ktoré sú aj hlavným faktorom, ovplyvňujúcim neiónickú difúziu látok cez bunkové membrány [3].

Tab. 1. Hodnoty clearance inulínu (C_{in}), clearance (C_{Ci}) a tubulárnej rezorpcie citrátu (R_{Ci}) u dojčiat

Por. číslo	Vek v mes.	C_{in}	C_{Ci}	R_{Ci} v %
		ml/mm/1,73 m²		
1	1	87,54	5,01	94,28
2	4	96,06	4,91	94,89
3	5	92,28	5,16	94,41
4	5	99,04	13,55	86,32
5	6	80,60	13,80	82,87
6	6	88,47	3,66	95,86
7	7	118,40	6,11	94,84
8	7	111,46	8,08	92,79
9	8	105,18	6,43	93,89
10	9	71,58	7,10	90,09
11	9	69,02	13,09	81,03
12	9	89,78	4,57	94,91
13	10	148,71	12,95	91,29
14	10	94,26	3,40	96,40
15	10	115,08	9,92	91,38
16	12	93,46	3,28	96,50
17	12	82,74	7,43	90,17
18	12	112,27	12,26	89,08
19	12	150,46	7,10	95,28
Priemer	8	100,33	7,78	91,91
Dospelí (1)			20,00	85,00

Clearance citrátu je výslednou hodnotou pôsobenia uvedených tubulárnych procesov, ktoré môžu byť v kvantitatívnom i kvalitatívnom zmysle značne ovplyvňované tak za fyziologických podmienok, ako aj pôsobením rôznych látok a za chorobných stavov. Najväčší význam sa pritom pripisuje hormónom, poruchám acidobázickej rovnováhy, D vitamínu, dietetickým zásahom, intermediátom Krebsovho cyklu, nefropatiám a viacerým chemikáliám [5]. Dôležitá úloha pripadá podľa Rechnenberga a spol. [7] aj veku jedinca.

U dospelých ľudí zistil Shorr a spol. (cit. podľa 1) vo 24-hod. vzorke moča clearance citrátu okolo 20 ml/min., čo zodpovedá v podstate aj nálezom pri krátkodobých sledovaniach Canaryho a spol. [1] a to 17 ml/min./100 ml glomerulárneho filtrátu (s rozmedzím 8—22 ml). Podľa týchto autorov predstavuje tubulárna rezorpcia hodnotu asi 85 % filtrovaného množstva citrátu (pri použití clearance endogénneho kreatinínu ako miery glomerulárnej filtrácie).

Hodnoty clearance citrátu, ktoré sme zistili u dojčiat vo 24-hod. vzorkách — ako aj pri krátkodobých odberoch moča — predstavujú sotva tretinu až polovicu

uvedených hodnôt dospelých ľudí. Tento rozdiel je podmienený hlavne značne vyššou tubulárnou rezorpciou citrátu v dojčenskom veku (temer 92 %). Rozdiel oproti údajom literatúry (1) vyplýva ale aj z rozdielneho spôsobu merania glomerulárnej filtrácie. Po prepočítaní na 100 ml glomerulárnej filtrácie, meranej pomocou clearance endogénneho kreatinínu, predstavovala nami zistená hodnota asi 63 % hodnoty, ktorú udávajú Canary a spol. (1) oproti 46 % pri použití inulínu ako miery glomerulárnej filtrácie. Ako u dospelých, tak aj u dojčiat je však endogénny kreatinín na presné meranie hodnoty glomerulárnej filtrácie málo vhodný. Hlavná podstata rozdielu medzi našimi výsledkami s údajmi literatúry (1) však asi spočíva vo zvláštnostiach metabolizmu v dojčenskom veku všeobecne a obličiek zvlášť.

Rozdiely medzi hodnotami clearance citrátu, ktoré sme získali z 24-hod. vzoriek moča a pri krátkodobých odberoch, môžu byť podmienené viacerými faktormi. Dôležitú rolu môže hrať čas odberu moča, pretože aj keď množstvo citrátu, vylúčené za 24 hodín, je relatívne stále, v priebehu dňa pomerne značne varíruje (2). Dôležité je aj uplatnenie rozdielnych faktorov, ktoré sa pomerne značne líšia pri odbere 24-hod. vzorky moča a pri použití clearanceovej techniky (1). Pri 24 hod. odbere moča sa uplatňuje zrejme aj vplyv prijímanej potravy a to nielen priamo, ale aj svojimi fyziologickými dôsledkami v oblasti acidobázickej rovnováhy a v zložení vnútorného prostredia (5).

Po zvýšení plazmatickej koncentrácie citrátu v priemere na 13,64 mg% nezistili sme existenciu maximálnej tubulárnej rezorpcie citrátu. Grollman a spol. (4) tiež nezistili (v pokusoch na psoch) existenciu maximálnej tubulárnej rezorpcie citrátu, a to ani pri zvýšení plazmatickej koncentrácie citrátu na toxické hodnoty.

Sledovanie účasti obličiek na metabolizme citrátu v dojčenskom veku môže byť jednou z ciest včasného zisťovania metabolických porúch všeobecne a zvlášť obličkového parenchymu. Okrem celkových porúch činnosti obličiek môžu pomôcť odhaliť poruchy regulácie acidobázickej rovnováhy, pretože exkrécia citrátu predstavuje aj obranný mechanizmus, pomocou ktorého môžu obličky šetriť fixné anionty. Tento obranný mechanizmus bol zistený už aj v novorodeneckom veku (6).

Súhrn

U 116 zdravých eutrofických dojčiat na ustálenom režime umelej výživy sme v 24-hod. vzorkách moča zistili priemernú hodnotu clearance citrátu 6,17 ± 2,58 ml/min./1,73 m² telesného povrchu.

Pri krátkodobých sledovaniach, robených ráno na lačno u dojčiat (vyberaných podľa rovnakých kritérií ako v prvej skupine), sme štandardnou clearanceovou technikou s použitím inulínu zistili priemernú hodnotu clearance citrátu 7,78 ± 3,68 ml/min./1,73 m² telesného povrchu. Priemerná hodnota tubulárnej rezorpcie vykazovala 0,026 ± 0,007 mg/1 ml glomerulárneho filtrátu, čo v relatívnych hodnotách predstavuje 91,91 ± 4,47 % hodnoty filtrovaného množstva.

Pri zvýšení citratémie v priemere 5,4-krát oproti kontrolnej hodnote sme nezistili existenciu maximálnej tubulárnej rezorpcie citrátu u dojčiat.

Тишлер В., Беньо П., Павковчекова О., Лахита И., Яцина Й.: **Почечный клиренс цитрата в грудном возрасте**

У 116 здоровых, автотрофических грудных детей, содержащихся на уравновешенном режиме искусственного питания, в суточных пробах мочи установлена средняя величина клиренса цитрата 6,17 ± 2,58 мл/мин/1,73 м² поверхности тела.

При кратковременных исследованиях, проводимых утром натощак у грудных детей (выбранных на основании тех же критериев, как и в первой группе) при помощи стандартной техники клиренса с применением инулина получены средние данные клиренса цитрата

7,78 ± 3,68 мл мин. 1,73 м² поверхности тела. Средние данные канальцевой резорбции составляли 0,026 ± 0,007 мг/1 мл клубочкового фильтрата, что в относительных данных представляет 91,91 ± 4,47 % фильтруемого количества.

При повышении цитратэмии в среднем в 5,4 раза, по сравнению с контрольной величиной, не удалось доказать существование максимальной канальцевой резорбции цитрата у грудных детей.

Cs. Pediat., 23, 1968, 6 : 492—496.

Tischler V., Beňo P., Pavkovčeková O., Lahita L., Jacina J.: **Renal Clearance of Citrate in Infancy**

An average citrate clearance value of 6,17 ± 2,58 ml./min./1,73 sq. m. of body surface was assessed in 24-hours samples of urine of 116 healthy normal infants receiving formula.

Samples taken in the morning in fasting infants chosen by the same criteria as in the first group showed the citrate clearance value (assessed by standard clearance technique with the use of inuline (7,78 ± 3,68 ml./min./1,73 sq. m. of body surface. The average value of tubular resorption was 0,026 ± 0,007 ml./ml. glomerular filtrate representing in relative values 91,91 ± 4,47 % of the filtered amount.

Though the citratemia was increased, on the average, to 5,4 times the control amount maximal tubular resorption of citrate in the infants was not observed.

Cs. Pediat., 23, 1968, 6 : 492—496.

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V. T., Košice, Moyzesova 9

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CANKER SORES FROM ALLERGY TO WEAK ORGANIC ACIDS (CITRIC AND ACETIC)

Case Report and Clinical Study

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ALLERGY to foods manifests itself in different ways and can give rise to a host of clinical manifestations. The etiological relationship between the latter and the food allergy may be definite in some instances, as for example, the development of angioedema of the lip and/or asthma following the ingestion of egg by an egg-sensitive child. That egg is responsible is indicated not only by the clinical history, but by the finding of a positive skin test reaction to egg and by the presence of circulating antibodies (reagins) to egg. Whenever it is possible to obtain such confirmatory evidence, the etiological association between the offending food and the clinical condition is easy to prove. But, unfortunately, this is not true of all the clinical manifestations ascribed to food allergy, especially if the skin test reaction to the suspected agent is negative. Under those circumstances, proof of an etiological relationship must depend entirely upon the subjective clinical data supplied by the patient after the offending food or foods are eliminated while on special or trial diets. One explanation offered for the occurrence of a negative skin test reaction to the food despite positive clinical tests is that the allergy exists to a split product of the protein (for example, amino acids, as in the case previously reported by us[1]), or to an enzyme (for example, that present in raw fruits or melons[2]), or to some other as yet undiscovered agent which does not give positive skin test reactions.

Recently, we saw a patient in whom recurrent outbreaks of canker sores, ascribed at times to food allergy, seemed to be associated with an abnormal reaction or allergy to weak organic acids like citric and acetic, as indicated by the results of our clinical studies. Since we could find no record in the literature of the demonstration of a similar etiological association, it seemed worth while to report this case history and the results of our clinical investigations.

CASE REPORT

A man, aged 37 years, an Army veteran, was first seen in the Outpatient Allergy Clinic of the Veterans Administration in May, 1953, complaining of nasal blockage, asthma, and headaches. These symptoms had begun at the age of 5, following an attack of whooping cough, and had been recurring since then. Little improvement was noted either after a tonsillectomy and adenoidectomy at the age of 7 or after a submucous resection done at the age of 19.

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The patient enlisted in the United States Air Force in January, 1942. He noted some increase in symptoms during his period of training and while flying planes in the China-Burma-India theater, especially as the result of changes in temperature as well as from exposure to the inhalation of soft coal smoke in southern Illinois and to volcanic dust in the Aleutians. In 1950, while in Texas and because of the persistence of symptoms, he was sent to the Brooks General Hospital for allergy investigation. Positive skin test reactions were obtained to milk and to a few other foods; also to dust, feathers, pyrethrum, and ragweed. Because of the recurrence of more severe symptoms of asthma and migraine headaches, he was sent to the Lackland Base Hospital where desensitizing injections of dust and ragweed pollen extracts were given for more than a year. Following this treatment, he noted fewer nasal and bronchial symptoms during the ragweed season, but continued to have headaches when exposed to dust or after the ingestion of certain foods, such as milk or chocolate.

For many years, probably since early childhood, the patient had frequent canker sores in the mouth. These were sometimes associated with headaches and also with vague muscular pains and varying degrees of lassitude. The canker sores persisted throughout his army life and up to the time he was first seen in our clinic. Having had them for so many years, he never complained about them and regarded them as trivial.

The patient remained in the Air Force until May, 1952, when he was discharged. In September, 1953, he enrolled at the University of Pennsylvania, in the Liberal Arts College, majoring first in psychology and later in sociology. Because of the continuance of the asthma, rhinitis, and headaches, he applied for treatment at the Philadelphia Allergy Clinic of the Veterans Administration. Complete intracutaneous skin tests revealed significant positive reactions to house dust, ragweed, timothy, and plantain, with negative reactions to the commonly eaten foods including milk and chocolate.

Treatment was begun shortly afterward. It consisted of the elimination of test-positive allergens and of desensitization with extracts of timothy and ragweed pollens and of house dust. The desensitizing injections still are being continued. The patient reports definite improvement in his nasal symptoms and in his asthma. The latter now is quite mild and is readily controlled by symptomatic measures (for instance, Tedral). Despite the institution of allergy treatment, the patient continued to have recurrent canker sores and headaches; also frequent spells of general lassitude, vague pains, irritability, and inability to concentrate, which interfered considerably with his studies. Because of these symptoms, he consulted the psychiatrist at the Veterans Clinic, who considered the symptoms functional in origin.

In attempting to correlate his symptoms with the offending allergen, the patient reported that exposure to smoke or dust of any type would induce severe nasal blockage and wheezing; ingestion of chocolate, candy, beer, nuts, and seafood also would cause nasal symptoms and, at times, asthma. Of particular interest in relation to our subsequent studies was his observation that the eating of chocolate (to which he had a negative intracutaneous skin test reaction) would provoke an outbreak of "sore spots" or canker sores in the mouth associated at times with "itchy bumps" on the scalp. The patient was quite accustomed to these canker sores, and for years had attributed them to the ingestion of sugars, sweets, "acid foods," or certain alcoholic drinks. The effect of the latter was verified by one episode recalled by the patient which took place during his tour of duty in Asia and which is of special interest in view of our subsequent clinical studies. Upon returning to the home base after a long tour of flying, the flight medical officer usually advised the pilots to take a few drinks to ease the tension and relieve the customary headaches which followed most flights. When the patient tried this, he usually developed migraine headaches, asthma, and canker sores. These symptoms at times were so severe that he could not report for duty, and he therefore seldom drank. He recalled that whenever he took mixed drinks containing fruit juices, he invariably developed symptoms; on the other hand, when he drank straight liquor, he might develop nausea associated with a mild "hangover," but would seldom get asthma, nasal blockage, or canker sores. The reasons for this difference will be evident later.

One of us (L. T.) saw the patient for the first time in May, 1954, in consultation. In reviewing the history with the patient, it seemed likely that the canker sores followed the ingestion of certain foods, especially grapes or anything made from grapes (for example, grape juice, jelly, wine, brandy); also, after eating chocolates and certain kinds of candies and carbonated beverages. To verify this possibility, we undertook further clinical studies and were fortunate in having the complete cooperation and exceptional assistance of a highly intelligent patient.

Clinical Studies.—A survey of the ingestants known clinically to produce his mouth symptoms indicated that one common factor nearly always was present, namely, citric acid. This substance was present either as a naturally occurring organic acid, as in citrus fruit, or it was used as an ingredient in the manufacture of commercially prepared products, as in carbonated beverages. The possibility that an organic acid such as citric acid could act as an allergen or could act as a mucous membrane irritant had never occurred to us and has not been noted in the allergy literature. Our attention to citric acid was drawn especially by some observations of this patient after a rather accidental experience.

Fig. 1.

Because of the restriction in diet, especially of milk, the patient felt weak and began to eat candy bars. This only seemed to make him worse. It induced heartburn, epigastric discomfort, gas, headache, canker sores, and general lassitude, and made concentration difficult. He attributed this to the chocolate and excess sugar and so tried other candies.

He began eating Necco wafers, especially while in class, and would take one of the wafers from the package in his pocket and slip it into his mouth without seeing it. He reported that certain wafers seemed to cause a puckering or itching sensation in the mouth and peculiar discomfort in the stomach. Upon further investigation, he noted that this would happen after eating yellow, green, or orange wafers which apparently contained citric acid, but not from the white, cinnamon, or even the chocolate-flavored ones, presumably those without citric acid. To eliminate the possibility that sugar was responsible, he ate pure cane sugar cubes and also rock candy without difficulty. Since he could take the chocolate wafer without trouble, he tried eating the pure chocolate which

is used by confectioners or bakers in preparing candy or pastry. This caused no symptoms, whereas most of the commercial chocolate bars, which according to the labels contained citric acid, induced canker sores.

This seemed to indicate that the patient was not allergic to chocolate itself but to substances combined with it. As a result of these experiences, the patient became quite adept at determining whether anything he ate or drank contained citric acid. Thus, for example, he found that he could not tolerate certain cough drops or troches (such as the red-colored Smith Brothers), whereas others were all right. This also was true of certain beverages; for example, he could drink unlimited amounts of Hires Root Beer (which, according to the label, does not contain citric acid) without symptoms, whereas one Coca-Cola or even club soda (both of which do contain citric acid) induced canker sores. As a result of these experiences, the patient became label conscious, inspecting all labels for citric acid and avoiding foods containing it. By strictly avoiding all substances containing citric acid, the patient could remain relatively free of canker sores for long periods and also could induce the sores at will by eating citric acid-containing foods.

Because of our suspicion that citric acid was the culprit, we coincidently began a series of investigations designed to determine whether our clinical suspicions could be verified. Since substances like citric acid do not give positive skin test reactions even in sensitive patients, we felt that there was a better chance of verification if we used a direct mucosal contact test. Accordingly, we had the patient put a small crystal of citric acid (contained in sour salt) into the oral cavity in the space formed by the lower lip and the lower anterior alveolar surface. Within a few hours, there was a reddening and edema at this area. By the next day a white, raised patch 12 to 18 mm. in diameter was present, with two or three papular elevations around its border. The area sloughed away leaving a craterlike ulcer with slightly raised edges (Fig. 1). Following the healing of this ulcer, which required about ten days, the patient then repeated the test in a different area, leaving the crystal in contact for fifteen seconds. Again the same chain of events occurred at the contact point, but a smaller area was involved. By later tests, it was found that even momentary contact of a citric acid crystal on the oral membranes was sufficient to produce canker sores. Control tests with potassium citrate crystals were negative, and tests with citric acid crystals upon members of the patient's family and many other individuals were negative. We then tried other related substances, or those containing citrate, with results shown in Table I. All the substances tested were in the form of crystals except magnesium citrate, which was in liquid form. The latter was tested by moistening a pledget of cotton with the solution and placing it in the oral cavity in the same manner as the crystals.

TABLE I. RESULTS OF MUCOSAL CONTACT TESTS WITH CITRIC ACID AND RELATED SUBSTANCES

SUBSTANCE	RESULT OF CONTACT TEST
Citric acid (sour salt)	Strongly positive
Potassium citrate	Negative
Magnesium citrate (solution)	Negative
Alka-Seltzer (citric acid, aspirin, sodium bicarbonate)	Moderately positive
Aspirin	Negative
Sodium bicarbonate	Negative
Bromo-Seltzer (citric acid, acetanilid, caffeine, sodium bicarbonate, sodium bromide)	Negative

It will be observed from Table I that all the compounds containing free citric acid gave positive mucosal tests with the exception of Bromo-Seltzer. The negative test to the latter perhaps can be explained by the fact that the citric acid in Bromo-Seltzer, when dissolved, quickly changes to sodium citrate. It is noteworthy that when the patient was

testing it, he noticed that when he placed the crystal against the oral mucosa it dissolved quickly in comparison with Alka-Seltzer crystals which remained in solid form much longer.

While it seemed evident from the results of our contact tests that citric acid was responsible for the canker sores in the clinical experiments, a question arose as to whether it was a matter of pH; also, even though the resultant ulcers were seen by one or both of us after the completion of some of the tests, observations of the patient himself had to be taken in reporting the results of most tests. To check this, blind mucosal tests were done by the patient with two solutions prepared by Dr. Hamilton* and marked "A" and "B." The patient was instructed to test the solutions by means of pledgets of cotton moistened in the solution and applied to the oral mucosa for a period of three minutes in the first test, and fifteen minutes in the later one. Different mucosal areas were used each time and sufficient time was allowed to elapse to permit the ensuing reaction to diminish. Solution "A" produced negative results both times. Solution "B", after three minutes, produced a definite positive reaction in the form of a red patch; after fifteen minutes, a much larger area of redness was noted, although no ulcer formed, probably because of the weak concentration used. Following the completion of these tests, we were told by Dr. Hamilton that Solution "A" was a weak solution of HCl (tenth normal), whereas Solution "B" was a weak solution (about 2.5 per cent) of citric acid in corresponding pH. These experiments, therefore, seemed to confirm the previous observations that citric acid was the specific offending agent, rather than the "acidity" or the irritative qualities of an acid substance.

TABLE II. RESULTS OF MUCOSAL CONTACT TESTS WITH ORGANIC ACIDS

SUBSTANCE	RESULT OF CONTACT TEST
Tartaric acid	Mildly positive
Acetic acid (apple vinegar)	Strongly positive
Lactic acid	Mildly positive
Uric acid	Negative
Ascorbic acid	Negative
Nicotinic acid	Negative
Benzoic acid (1%)	Negative

The question next arose as to whether other organic acids might act in a similar fashion to citric acid. After consultation with Dr. Hamilton, a series of mucosal tests, done either with the pure crystals or with a solution soaked in a cotton pledget, were carried out by the patient with those organic acids most frequently found in the human diet. The results of these tests are summarized in Table II.

The marked positive mucosal reaction to the application of acetic acid was surprising. But, at the same time, it explained why the patient had mouth ulcers and gastrointestinal symptoms after eating foods containing vinegar; thus, for example, the patient reported that he would develop a typical right-sided migraine headache as well as canker sores and itching of the skin after eating foods containing vinegar, such as cole slaw, potato salad, or beef cooked in vinegar, whereas he could eat plain beef with impunity. These symptoms were similar to those he noted after taking foods with excess citric acid (for example, fruit juice) but seemed more intense perhaps because he took larger amounts.

Another question presenting itself for further investigation was whether the organic acids, like citric acid, acted by direct contact with the buccal mucosa or after ingestion or by both means. To determine this, the patient took a 3 grain (0.2 Gm.) capsule (washed beforehand) of citric acid shortly after breakfast. He had no immediate reaction; but several hours later noted a gnawing pain in the epigastrium, mild diarrhea, and

*The authors are greatly indebted to Robert H. Hamilton, M.D., Professor of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania, for his invaluable advice and assistance in carrying out some of the biochemical studies and for some of the material employed in the tests.

slightly irritated tongue. A month later (December, 1954), he took a 5 grain (0.3 Gm.) capsule of citric acid after each of his three meals for two days, but noticed no untoward effects. Despite these results, the patient observed that ingestion of foods containing excess citric acid (fruit juices or wine) produced not only canker sores but at times inflamed furuncle-like lesions usually in the nares and less commonly in the lower lid (resembling a stye), mostly on the right side. This would seem to indicate that the organic acids act not only after direct contact but also after getting into the stomach and intestines, depending upon the amount ingested and the amount absorbed into the circulation.

Further clinical trials were made with other foods, especially fresh fruits, to determine whether they would induce the formation of canker sores. As a result of these tests, it was determined that canker sores could be provoked not only by commercial foods or drugs containing citric or acetic acids but also after the ingestion of the following foods: oranges, grapes, dried apricots, raw and canned peaches, pineapple, wines especially Chianti, brandies, beer, and most mixed alcoholic drinks. On the other hand, he determined that he could eat with impunity the following: raw or cooked apple, raw or canned pears, canteloupe, watermelon, honeydew melon, Persian melon, and raw celery; however, canned celery juice caused a puckering sensation in his mouth, perhaps because of the added preservative. While most of the reacting foods induced canker sore formation, this was not true of all of them. Thus, ingestion of milk or cheese precipitated headache, malaise, and wheezing, but no canker sores.

Following the diagnostic study and especially after extensive clinical trials which revealed the offending agents, the patient eliminated as far as possible all offending foods as well as those containing citric acids or acetic acids. As a consequence, he has noticed definite lessening of his allergic symptoms except when he inadvertently or consciously is indiscreet in his diet; if so, he can easily detect the cause of his symptoms in retrospect; or if it is a canned food, he later finds that the food contained citric acid of which he previously was unaware. He also has noted definite improvement in his general physical and mental condition, especially in his power of concentration and in a lessening of his previous irritability. The latter condition was the basis for his periodic visits to the Clinic psychiatrist for two years, but he noted no beneficial effect upon his symptoms until after the offending foods were eliminated. He now sees the Clinic psychotherapeutist (psychologist) periodically for assistance in his school problems.

COMMENT

The clinical study of this patient has revealed many interesting features which might find application to other similar patients. In the first place, it was demonstrated that the patient had a definitely abnormal reaction or allergy to citric acid and to foods or drugs containing it. This reaction took the form of canker sores proved by direct mucosal contact tests with citric acid in this patient, whereas the same agent in other control patients gave negative reactions. Similar abnormal reactions were observed from other organic acids, notably acetic acid. One might question the use of the term *allergy* to designate a reaction to nonantigenic substances like citric or acetic acids, but similar abnormal reactions designated *allergic* occur in relation to various nonprotein agents (for instance, drugs) which likewise give neither a positive skin test reaction nor demonstrable antibody. Regardless of the designation, our demonstration in this patient, apparently for the first time, of an allergy to weak organic acids is of great importance in food allergy. It is another link in the chain of evidence indicating that allergic reactions to foods need not necessarily occur in relation to the protein component but may take place in relation to the split products like peptones and amino acids, or

preformed weak organic acids, and possibly to organic acids formed from substances in food other than proteins. Such patients may have negative skin test reactions to the parent food and yet possess an allergy (of the nonatopic type) to one of its products. This helps to explain those instances where the patient has a clinical allergy to a food and yet gives a negative skin test reaction to its extract. Another interesting feature in this patient is the fact that we were able to prove by direct mucosal tests that the canker sores were caused by the abnormal reaction to the weak organic acids. Similar testing with citric acid crystals and/or a weak solution of acetic acid might be carried out in those allergic patients in whom food allergy is suspected but the skin test reactions are negative, as for example in patients with canker sores.

Despite the clinical demonstration in this patient that contact with weak organic acids induced the formation of canker sores, the manner by which this is brought about raises many unexplained questions. First is the matter of specificity. Thus, canker sores were produced in this patient not only by citric acid but also by acetic acid and, to a mild degree, by tartaric and lactic acids. Likewise, it is not easy to explain, for example, why the canker sores were induced by contact with citric acid but not from sodium citrate. The evidence needed to answer these questions involve such matters as pH, concentration, solubility, or the buffer powers of organic acids compared with that of weak HCl and can be furnished only by further experimental investigation which is now in progress.

The fact that canker sores in this patient were proved to be due to allergy to citric acid and acetic acids is of interest also because of the popular custom of physicians and patients to attribute these canker sores to an excessive ingestion of "acid foods," especially fruit juices. Suspicion of an etiological relationship could not always be verified, except for a lessened tendency for the ulcers to develop when the suspected foods were eliminated. Our investigations furnish greater support to what previously seemed like mere suspicion and indicates that "acid foods" actually can have a causal relationship. They also explain the tendency of the patient to get canker sores when he drank mixed drinks containing fruit juices and not from straight liquors.

Finally, the clinical history in this patient demonstrates that symptoms regarded as functional or psychosomatic sometimes may be due to an unsuspected or unproved food allergy. Thus, in the case herein reported, symptoms of "toxemia," including headache, muscular neuralgic pains, lassitude, irritability, and inability to concentrate, improved considerably after the elimination of the offending foods, including those containing citric acid and acetic acid, and reappeared after their readdition. This is not new, for Rowe,[3] many years ago, included these symptoms in a syndrome designated by him as "allergic toxemia," but its existence in some patients sometimes is forgotten in our present emphasis upon functional causes.

SUMMARY

A report has been made of a male patient, aged 37, treated for nasal allergy, asthma, and migraine who for years was subject to frequent outbreaks

of oral canker sores. Clinical investigation indicated that the latter usually followed the ingestion of foods, drugs, or carbonated beverages containing citric acid. Direct application of citric acid crystals to the oral mucosa repeatedly reproduced the ulcer in the patient, but not in the controls. Mucous membrane contact tests with substances containing citric acid gave similar positive results; likewise, tests with other weak organic acids contained in foods gave positive reactions to some of these, especially to acetic acid. Avoidance of foods containing the positive reactors was followed by marked relief not only of the ulcers but also of the general "toxic" symptoms previously regarded as functional. This demonstration of possible allergic reactions to weak organic acids in foods provides additional explanation for the existence of clinical allergy to certain foods in some patients despite the presence of a negative skin test reaction.

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Acute and Subacute Toxicological Studies of TAKEDA-Citric Acid in Mice and Rats

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Summary

The acute and subacute toxicities of TAKEDA-citric acid were examined in mice and rats. The large single doses administered orally, subcutaneously or intraperitoneally in mice produced some behavioral changes and death by respiratory or cardiac failure. Though the high daily oral doses for six weeks in the rat produced some but minor biochemical changes of blood and urine, no specific pathohistological abnormality was detected in the organs.

Introduction

TAKEDA-citric acid (NPC) is a refined final product of citric acid, produced by the fermentative procedure of yeast belonging to the *genus Candida* using normal paraffins as nutrient carbon source (Belgian patent 716247 (July 31, 1968)). The intraperitoneal LD_{50}s of commercial citric acid in mice and rats are shown to be 5.0 (961 mg as anhydrous) and 4.6 millimole/kg (884 mg/kg), respectively[1]. Daily oral administration of 600 mg/kg (1.2% in the diet) for more than 90 days to rats is reported to produce no abnormalities in body weight gain, blood, pathohistology of the viscera, and reproduction[2]. Also, daily oral administration of 1380 mg/kg of citric acid to dogs for 112 to 120 days is shown to produce no behavioral, biochemical, or pathohistological abnormalities[3]. In the present study, the acute toxicity of NPC in mice in comparison with that of commercial citric acid and its subacute oral toxicity for 6 weeks in rats were examined in order to confirm the safety.

Methods

The purity of NPC (Lot No. M_1-100) used was 99.8% as citric acid monohydrate. Heavy metals and normal paraffins were present at the concentration below 0.001% and below 0.1 ppm, respectively. Also 3,4 benz[α]pyrene was not detected by a modified method of Howard *et al.*[4], in which 0.02 ppb of this compound could be analyzed[4]. Commercially available citric acid was used as positive control in the acute toxicity test.

In the acute toxicity test, 4-week old male ICR-JCL mice, weighing 20 to 24 g, and 5-week old male SD-JCL rats, weighing 110 to 140 g were used. The animals were kept in an air-conditioned room allowing free access to a commercial diet (CLEA CE 2) and drinking water. The solutions of test compounds in desired concentrations were administered subcutaneously, intraperitoneally and orally in volumes of 0.5 ml/10 g body weight and 2 ml/100 g body weight to mice and rats, respectively. The number

Subcutaneous injection: Mice and rats exhibited behavioral signs similar to that with the oral and intraperitoneal routes. A few minutes after administration the animals showed mydriasis and decrease in rate of respiration, and thereafter laid down on their side. Death in animal of both species given each dosage of NPC occurred within 15 minutes to 6 days by respiration failure or emaciation. In animals given high doses, considerably marked necrosis of the skin and inflamation at the injection site were morphologically observed. Similar but a milder inflammatory process at the injection site was found in animals given low doses.

LD_{50}s were shown in Table I.

Table I. Acute Toxicity of NPC and Citric Acid in Male Mice and Rats

Samples	Species	Rute of administration	LD_{50}, mg/kg[a] (95% confidence limits)
NPC	Mouse	*p.o.*	5790 (4990– 6720)
		i.p.	940 (872– 1019)
		s.c.	2700 (2390– 3050)
	Rat	*p.o.*	11700 (10080–13570)
		i.p.	725 (690– 762)
		s.c.	5500 (4700 – 6435)
Citric acid[b]	Mouse	*p.o.*	5040 (4520 – 5665)
		i.p.	960 (888 – 1037)

a: Method of Litchfield and Wilcoxon.
b: Commercial citric acid (special grade).

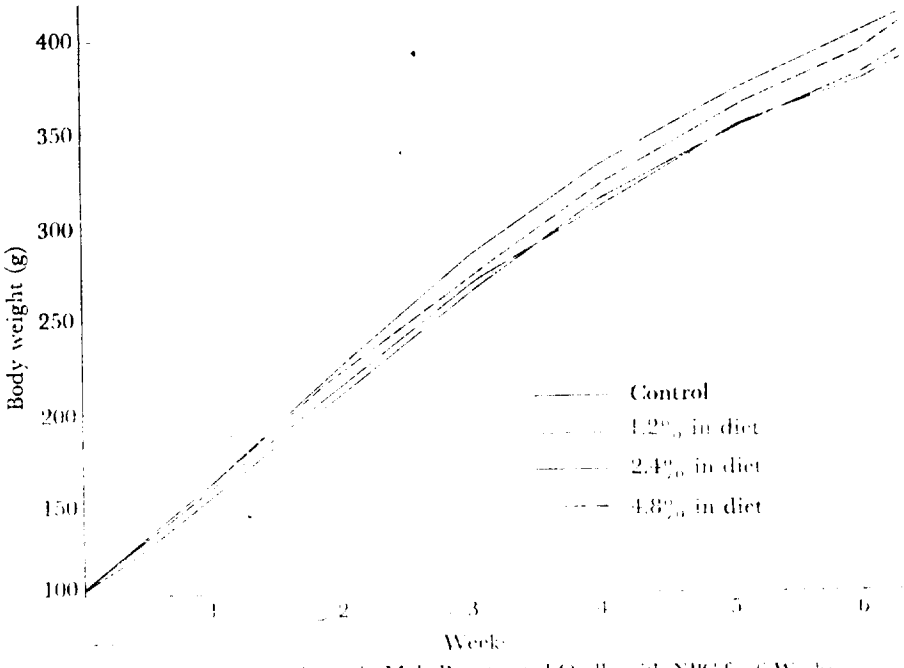

Fig. 1. Mean Growth Curves in Male Rats treated Orally with NPC for 6 Weeks

II. Subacute toxicity

i. Behavioral effect: No treated animals exhibited behavioral abnormalities throughout the feeding period. The body-weight gain in animals fed 1.2, 2.4 and 4.8% NPC diet was found to be depressed slightly from the end of 1 or 2 weeks and from the beginning of 1 week, respectively. The percent body weight gain in animals with the respective feeding were 98.8 92.6 and 94.0% of that of the control group, respectively fed at the termination (Fig. 1). The daily food-intake/100 g body weight in animals fed 1.2, 2.4, and 4.8% NPC diet was depressed by 0.7, 2.6 and 4%, respectively, of the levels in the control animals. The mean daily NPC-intake measured in the respective groups were 1.15, 2.26 and 4.67 g/kg/day.

ii. Morphological and biochemical examinations of blood and urine: Slight but not significant decrease in the counts of erythrocytes and leucocytes, hematocrit value, and hemoglobin content were detected in the animal groups given higher doses. Analysis of the hemogram revealed only relative decrease in lymphocytes and the relative increase in neutrophile granulocytes (Table II).

Table II. Blood Picture in Male Rats treated Oraliy with NPC for 6 Weeks

Dosage % in diet	No. of rats	Erythrocytes ($\times 10^4$/mm^3)	Hematocrit (%)	Hemoglobin (g/dl)	Leucocytes ($\times 10^3$/mm^3)	Differentials				
						Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
0	5	768.0[a] ±19.2	46.6 ±0.9	16.1 ±0.5	8.42 ±1.29	6.8	90.4	2.6	0.2	0
1.2	5	766.0 ±27.0	45.4 ±1.5	16.1 ±0.8	8.20 ±1.09	3.4	93.8	2.6	0.2	0
2.4	5	738.0 ±61.4	45.0 ±3.5	15.8 ±0.8	8.02 ±2.12	10.4	85.4	3.6	0.6	0
4.8	5	730.0 ±60.0	44.2 ±3.8	15.3 ±1.5	7.88 ±1.97	10.8	85.8	2.8	0.6	0

a: Mean ± S.D.

The total plasma protein tended to decrease in the treated animals and the same decrease in the 2.4% groups was statistically significant. In addition, the decrease in the plasma albumin and the ratio of albumin/globulin in the plasma were found only in animals treated with the 4.8% NPC diet. The highest dosage group exhibited some decrease in the plasma cholesterol level and elevated activity of GOT in the serum. Some of the animals given higher doses showed a slightly prolonged BSP retention. The blood level of calcium ion in the treated animals showed some decrease, which proved to be not either significant or dose-dependent (Table III).

One of the animals fed the 4.8% NPC diet showed an increased presence of protein in the urine, and the occult blood reaction was demonstrated in 4 of 5 animals fed the 4.8% NPC diet. Many lymphocyte-like cells were detected in the sediment of the urine with the increased presence of protein.

iii Pathohistological examinations: Abnormality in the size of the testis was found in two animals fed the 1.2 and 4.8% NPC diet, respectively. The other macroscopical

Table III. Results of Biochemical Examination in Male Rats treated Orally with NPC for 6 Weeks

Dosage % in diet	No. of rats	T. prot. (g%)	Alb. (g%)	Glob. (g%)	A/G ratio	Ca^{++} (mg%)	T. chol. (mg%)	Glu. (mg%)	BUN (mg%)	Creat. (mg%)	T. bili. (mg%)	Alk. phos. (mU/ml)	LDH (mU/ml)	GOT (mU/ml)	GPT (R.F.U./ml)	BSP retention (mg%)
0	5	6.51[a] ±0.17	3.01 ±0.53	3.49 ±0.38	0.88 ±0.26	10.05 ±0.31	77.8 ± 6.0	120.4 ±14.9	13.9 ± 2.4	0.53 ±0.06	0.12 ±0.04	153.0 ±33.3	113.4 ±13.5	143.4 ±22.3	39.4 ± 3.3	0.32[(3)c] ±0.05
1.2	5	6.26 ±0.18	2.62 ±0.06	3.64 ±0.16	0.72 ±0.03	9.65 ±0.51	75.0 ±10.8	117.8 ± 8.2	15.4 ± 1.3	0.55 0.03	0.10 ±0.01	183.6 ±16.5	119.0 ±17.3	162.0 ±12.7	40.6 ± 4.9	0.34[(3)] ±0.09
2.4	5	6.16[b*] ±0.22	2.86 ±0.32	3.29 ±0.40	0.89 ±0.23	9.62 ±0.35	75.4 ± 6.2	140.4 ±15.0	13.5 ± 1.6	0.53 ±0.06	0.10 ±0	176.4 ±22.2	113.0 ±14.4	164.4 ±11.2	43.0 ± 2.7	0.41[(3)] ±0.08
4.8	5	6.22 ±0.32	2.80[d] ±0.53	3.42 ±0.51	0.85[d] ±0.29	9.69 ±0.45	70.2* ± 1.8	125.2 ±17.7	12.9 ± 1.2	0.59 ±0.07	0.10 ±0.01	166.4 ± 4.9	137.6 ±37.1	175.0* ±16.2	44.0 ± 4.2	0.38[(8)] ±0.11

a: Mean ± S.D.
b: Significantly different from control, *$P<0.05$.
c: No. of rats used.
d: One of animals: 1.95 g% (Alb.) and 0.49 (A/G ratio).

Table IV. Mean Organ Wet Weights in Male Rats treated Orally with NPC for 6 Weeks

	Dosage % in diet	No. of rats	Final B. wt. (g)	Carcass (g)	Carcass (%)	Brain (g)	Heart (g)	Lungs (g)	Liver (g)
Absolute	0	8	409.0[a] ±19.2	312.9 ±18.9	76.8 ±1.7	2.14 ±0.08	1.29 ±0.11	1.38 ±0.09	12.24 ±2.37
	1.2	8	405.4 ±14.0	309.5 ±15.0	76.3 ±2.1	2.14 ±0.09	1.21 ±0.09	1.38 ±0.07	12.13 ±2.27
	2.4	8	391.2 ±23.4	297.2 ±18.4	76.0 ±1.7	2.07 ±0.07	1.25 ±0.22	1.39 ±0.15	12.36 ±3.38
	4.8	8	390.5 ±23.1	295.5 ±21.5	75.6 ±2.5	2.03* ±0.10	1.23 ±0.09	1.39 ±0.15	11.70 ±2.43
Relative	0	8	100	76.8		0.52	0.31	0.34	2.99
	1.2	8	100	76.3		0.53	0.30	0.34	3.00
	2.4	8	100	76.0		0.53	0.32	0.35	3.16
	4.8	8	100	75.6		0.52	0.31	0.35	3.00

	Dosage % in diet	No. of rats	Kidneys R (g)	Kidneys L (g)	Spleen (g)	Testes R (g)	Testes L (g)	Prostate (g)	Adrenal glands (mg)	Thyroid (mg)	Hypophysis (mg)	Thymus (g)
Absolnte	0	8	1.48 ±0.12	1.50 ±0.14	0.76 ±0.11	1.49 ±0.14	1.49 ±0.12	0.63 ±0.12	56.1 ± 9.3	16.6 ± 3.2	13.3 ± 1.7	0.68 ±0.11
	1.2	8	1.46 ±0.18	1.43 ±0.14	0.75 ±0.11	1.57 ±0.62	1.40 ±0.22	0.85*[b] ±0.22	55.4 ± 8.2	16.5 ± 3.6	13.4 ± 1.3	0.63 ±0.08
	2.4	8	1.40 ±0.17	1.40[6][c] ±0.18	0.71 ±0.10	1.53 ±0.06	1.53[6] ±0.07	0.60 ±0.16	54.6 ±9.8	15.6[6] ± 2.6	12.7 ± 1.5	0.68 ±0.19
	4.8	8	1.45 ±0.20	1.51[6] ±0.19	0.68 ±0.14	1.45 ±0.36	1.57[5] ±0.07	0.65 ±0.18	54.4 ± 9.8	17.1[6] ± 1.9	12.7 ± 1.7	0.53* ±0.10
Rolative	0	8	0.36	0.37	0.18	0.36	0.36	0.15	13.7	4.0	3.2	0.17
	1.2	8	0.36	0.35	0.18	0.39	0.34	0.21	13.7	4.1	3.3	0.15
	2.4	8	0.36	0.35[6]	0.18	0.39	0.39[6]	0.15	13.9	3.9[7]	3.2	0.17
	4.8	8	0.37	0.38[6]	0.17	0.37	0.39[5]	0.17	13.9	4.4[7]	3.2	0.13

R: Right, L: Left.
a: Mean±S.D.
b: Significantly different from control, *$P<0.05$.
c: No. of rats used.

abnormality was yellowish-white miliary tubercles on the inner surface of the right hepatic lobe. The thymus and the spleen of animals fed the 4.8% NPC diet showed some decline in wegiht and the decrease in thymus weight was significant. The prostate in animals fed the 1.2% NPC diet and the thyroid gland in animals fed the 4.8% NPC diet showed some decrease in weight (Table IV).

Slight atrophy of the cortex of the thymus and of the lymph follicle of the spleen in animals fed the 4.8% NPC diet was observed histologically. However, other histopathological lesions especially those reflecting a decrease in organ weight, were found in the organs of any of the rats fed the 1.2, 2.4 and 4.8% NPC diet.

Discussion

Citric acid is one of the metabolites of the TCA cycle in the body and participates in the activity of acetylcoenzyme A carboxylase, formation of bone, and metabolism of parathyroid hormone. The decalcifying effect of citric acid interferes with blood clotting. However, daily oral administration of NPC by mixing in the diet in ratios of 1.2, 2.4 and 4.8% for 6 weeks produced no behavioral abnormalities, except a slight depression of body weight gain and daily food-intake. Morphological and biochemical examinations of the blood and urine demonstrated some but mild abnormal findings. However, many of the findings could not be ascribed to the toxic effect of NPC. Though the blood levels of calcium ion in the treated animals decreased slightly, the decrease was not dose-dependent. The urine is normally calcium-free, but calcium rapidly appears in the urine when the plasma concentration is elevated by even a slight degree. The presence of occult blood in the urine of rats given the highest dose of NPC suggests an increased output of calcium salts. However, no evidence to support the suggestion was presented in the present experiment. Also no deleterious effects of NPC on organs were observed histologically under the present experimental conditions.

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EFFECT OF ADMINISTRATION OF THIAMINE TETRAHYDRO-FURFURYLDISULFIDE AND CITRIC ACID ON THE URINARY VITAMIN C AND OXALIC ACID EXCRETION

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There have been quite many reports on the utilization[1] of vitamin C and the related oxalic acid utilization. Oxalic acid had been considered an end product of vitamin C utilization in the human body, but now it is said that vitamin C is also used as an energy resource. Oxalic acid in blood and urine is medically and biochemically significant. It increases in cirrhosis, beriberi, and supportative diseases, accumulates in blood in cases of neuralgia or rheumatism,[1] and also influences renal diseases and urinary calculus. It is known that ingestion of vitamin B_1 and C gives a favorable result to the patients of the above diseases.

In a living body, sugar and glycine produce oxalic acid, and citric acid produces oxalic acid and acetic acid. These are excreted in urine. Oxalic acid produced by utilization of vitamin C exists as $NH_2CONHCOCOOH$ combined with urea.[2] Citric acid, an element in oxalic acid production, is a middle product of sugar utilization, a regular element in a body and an important compound in the Krebs Circuit. Vitamin B_1 is necessary in the process of citric acid production from pyruvic acid and deficiency of vitamin B_1 decreases citric acid. Thus oxalic acid and citric acid are deeply related to sugar utilization, and vitamins C and B_1 are also significantly related to each other.

The writer reported before that a positive correlation is seen between the amount of orally injected vitamin C and urinary oxalic acid excretion and also that the amount of oxalic acid decreases during a female menstrual period.(3)

Noting the relationships between citric acid and oxalic acid and between vitamins C and B_1, we studied the effect of oral administration of TTFD and citric acid on the urinary vitamin C and oxalic acid excretions.

Methods

(1) Examinees

The examinees were two 18 year-old females, one 19 year-old female, and one 36 year-old male. S.N. has light anemia, S.F. light arthritis, and both R.Y. and Y.Y. are healthy, but the latter is a heavy tea drinker.

(2) Urine collection

Urine was collected at 10:30 am and 12:30 pm, to be tested immediately.

(3) Test period

Four days as one unit between June 23 and July 17, 1967.

(4) Administration of TTFD and citric acid

TTFD (50 mg) and citric acid (5 mg citric acid dissolved in 200 ml water containing 30 g cane sugar) were administered at 9:00 am, since urinary vitamin C excretion increases around 2 or 3 pm affected by breakfast or lunch.

(5) Oxalic acid measurement

Bergorman Elliot Method(3) was used. Colors were compared at 515 mμ by Hitachi spectometer Model 101.

(6) Vitamin C measurement

Colors were comapred at 530 mμ by the above spectrometer using Indophenal Method by Sato and others and Phenylhydrazine Method by Fujita and Temuchi.

Test Results

(1) Urine and pH

Average urine excretion of each examinee was 69, 57, 23, and 104 ml respectively before the experiment; 67, 63, 32, and 100 ml at the time of cane sugar addition; 71, 58, 30, and 92 ml at the time of TTFD administration; and 56, 62, 34, and 102 ml at the time of citric acid administration. R.Y. excretes very little urine and Y.Y. excretes as much as 100 ml. Administration of TTFD, citric acid and cane sugar did not change urine excretion. pH was 5.1 to 7.2 and no change was found.

(2) Urinary vitamin C and oxalic acid exretion before the experiment.

Table 1 shows urine excretion of each examinee before the experiment. The measurement was taken by Phenylhydrazine Method during the first two days and by Indophenol Method during the last two days of the four day experiment. The following tests were all done in the same way.

The average vitamin C excretion of S.N. was 3.58 mg % while S.F. showed as much as 5.60 mg %, 1.7 times as large as S.N.'s measurement. R.Y. and Y.Y. showed 5.15 and 5.53 mg % respectively. Y.Y.'s heavy tea drinking seems to have caused a considerably large amount of vitamin C excretion. It is said that in general anemia patients have low vitamin C which plays an important role in the production of erythrocytes in the medulla and also that a chronic vitamin C deficiency causes low blood-iron or anemia for which an iron preparation does not work well without vitamin C administration. The case of S.N. seems to suggest the above.

Table 1 Urinary Vitamin C Excretion (mg%)

試験日 A	採尿回 B	S.N.	1:2 比 C	S.F.	1:2 比 C	R.Y.	1:2 比 C	Y.Y.	1:2 比 C
6. 23	1 *	2.59	0.81	3.12	0.93	2.73	1.03	3.47	1.01
	2 **	2.19		2.90		2.81		3.51	
24	1	3.00	1.00	1.29	0.93	3.81	1.05	4.51	0.93
	2	3.00		4.01		1.00		1.22	
25	1	1.66	0.81	7.70	0.99	7.15	0.88	7.64	0.82
	2	1.00		7.65		6.27		6.30	
26	1	4.79	0.91	7.57	1.00	6.98	1.03	7.78	0.87
	2	1.10		7.60		7.22		6.80	
	平均 D	3.58	0.89	5.60	0.96	5.15	0.99	5.51	0.91

* 10時30分採尿　　** 12時30分採尿

A: Date
B: # of urine collection
C: Porportion
D: Average

*: Collected at 10:30
**: Collected at 12:30

Table 2 shows the urinary oxalic acid excretion. S.N[5] showed 3.3mg%, a considerably low value compared with the others and Y. Y showed a high value, 5..5mg%. There seems to be a positive correlation between the urinary vitamin C and oxalic acid excretion.

Table 2 Urinary Oxalic Acid Excretion (mg%)

A 試験日	B 採尿回	S.N.	1:2 比C	S.F.	1:2 比C	R.Y.	1:2 比C	Y.Y.	1:2 比C
6. 23	1	2.0	1.30	4.7	0.85	4.2	0.95	4.2	0.93
	2	2.6		4.0		4.0		3.9	
24	1	3.4	1.25	5.1	0.94	4.8	0.91	6.1	0.95
	2	4.4		4.8		4.5		5.8	
25	1	3.0	1.30	4.9	0.82	7.1	0.93	7.2	0.70
	2	3.9		4.1		6.9		5.0	
26	1	3.8	0.93	5.3	0.94	5.1	0.90	4.9	0.82
	2	3.6		5.0		4.6		4.1	
	D 平均	3.3	1.20	4.7	0.89	5.2	0.93	5.15	0.85

A: Date
B: # of urine collection
C: Proportion
D: Average

Table 3 Urinary Vitamin C Excretion (mg%) at the Time of TTFD Administration

A 試験日	B 採尿回	S.N.	1:2 比C	S.F.	1:2 比C	R.Y.	1:2 比C	Y.Y.	1:2 比C
6. 30	1	2.1	0.52	5.4	0.70	3.9	0.80	4.7	0.80
	2	1.1		3.6		3.0		3.7	
7. 1	1	3.0	0.90	4.3	0.84	3.8	1.00	5.0	1.00
	2	2.7		3.5		3.1		5.0	
2	1	4.9	0.90	7.9	0.76	7.4	0.80	7.7	0.80
	2	4.4		6.0		6.2		5.9	
3	1	5.1	0.96	7.6	0.80	7.0	0.72	7.8	0.72
	2	4.9		5.9		6.4		5.6	
	D 平均	3.5	0.82	5.5	0.77	5.1	0.83	5.6	0.83

of animals in the respective dosage groups was 6. Behavior and mortality were observed for 7 days after administration, and the LD_{50}s were calculated by the method of Litchfield and Wilcoxon.

Male SD-JCL rats weighing 98 to 112 g and 29 to 35 days old at the beginning of feeding trial were used in the subacute toxicity experiments. They were divided into 4 groups each consisting of 10 animals, and maintained on a powdered commercial diet (CLEA CE 2) and drinking water were given ad libitum and the room temperature was maintained at 23±1° with humidity of 55±5%. Five animals were placed in each one metal cage and administered the test agent orally by mixing in the diet at rates of 0, 0.2, 2.4 and 4.8%, respectively.

The parameters observed were body weight measured individually once or two times a week, daily food-intake measured in a group of 5 animals two times a week, behavioral abnormality observed every day, and urine examination individually on 41th feeding day. On the last feeding day, the BSP test wasp erformed. After fasting for 24 hours, the blood sampled from the abdominal artery of the animals slightly anesthetized by ether inhalation was subjected to morphological and biochemical examinations. Thereafter, the animals were sacrificed by exsanguination and the organs were weighed and fixed in 10% formalin for preparation of histological specimens stained with hematoxylin-eosin.

Results

I. Acute toxicity

Oral administration: Several minutes after administration the spontaneous movement of mice and rats within the cage was observed to be activated. Fifty minutes, thereafter, they showed motor ataxia, and then laid down on their side. Mydriasis and decrease in rate of respiration and heart beat were observed. Death of animals given 5790 or 7000 mg/kg (mice) and 12500 or 18000 mg/kg (rats) occurred within 20 to 180 minutes by respiratory failure. One animal given 4820 mg/kg (mice) or 10420 mg/kg (rats) of NPC died respectively at 20 hours. The animals which survived from the respiratory failure began to recover gradually within several hours and showed no toxic signs 24 hours later. No abnormalities were found at autopsy except the presence of the hemorrage of the gastric mucosa. LD_{50}s and behavioral signs of almost the same order agents were exhibited by both agents.

Intraperitoneal injection: Immediately after administration the animals showed stretching and slow crawling thereafter some laid down on their side. Death of mice given 1250 mg/kg of test compounds occurred within 6 to 180 minutes by respiratory and cardiac failures. Some of mice and rats given doses below 1042 mg/kg of NPC survived but showed tremor, slight opisthotonus and a slow avoidance reaction to external stimuli within 30 minutes after administration. Some of the animals died by emaciation 2 to 5 days after injection, while others were found to recover within 3 days. At autopsy the surface of the spleen and liver was found to be covered with a thin whitish membrane and there was slight hypertrophy of liver. No differences were observed in LD_{50} and behavioral signs of both agents.

A: Date
B: # of urine collection
C: Proportion
D: Average

(3) Effect of TTFD administration on urinary vitamin C and oxalic acid excretion

Table 3 showss the urinary vitamin C excretion at the time of 50mg TTFD administration. Vitamin C excretion of four examinees was 3.5, 5.5, 5.1 and 5.6mg%. S.N, S.F and R.Y showed decrease compared with before the administration. The 2% decrease of excretion by the administration of TTFD indicates that TTFD administration improved metabolism which used more vitamin C in deoxidization.

We did vitamin B_1 load test before this experiment. Urinary vitamin B_1 excretion 24 hours before the B_1 administration was 60, 70, 76 and 110 g respectively and the rate of increase of urinary vitamin B_1 excretion 24 hours after 5mg B_1 administration was 7, 9, 12 and 11%. The above results indicate that S.N already had a vitamin B_1 deficiency, S.F lacks vitamin B_1 to a small extent and the other two had normal intake of vitamin B_1. Therefore the decrease of urinary vitamin C excretion of S.N and S.F can be considered as the result of improved metabolism.

Table 4 shows that TTFD administration also increases urinary oxalic acid excretion. S.N, S.F and Y.Y showed

24, 9 and 8% increase respectively, but R.Y showed 2% decrease. This indicates that TTFD administration generally promotes oxalic acid excretion.

Table 4 Urinary Oxalic Acid Excretion (mg%) at the time of TTFD Administration

A	B	S.N.	1:2 C	S.F.	1:2 C	R.Y.	1:2 C	Y.Y.	1:2 C
6.30	1	1.2	1.10	5.3	0.73	4.9	0.96	5.5	0.62
	2	1.6		3.9		1.7		3.1	
7.1	1	1.1	1.10	5.9	0.97	5.2	0.96	7.7	0.90
	2	1.9		5.7		5.1		7.0	
		1.0	1.20	5.0	1.02	5.3	1.00	5.6	0.71
		1.0		5.1		5.3		1.0	
	1	1.2	0.93	5.3	0.80	5.5	0.90	6.0	0.90
	2	2.0		1.7		1.9		5.1	
	D	1.2	1.08	5.1	0.89	5.1	0.95	5.6	0.78

A: Date
B: # of urine collection
C: Proportion
D: Average

(4) Effect of administration of citric acid on urinary vitamin C and oxalic acid excretion

As shown in Table 5, urinary vitamin C excretion of the examinees increased to 6.6, 5,6, 5,8 and 6.3mg% compared with the time of TTFD administration. Especially the excretion of S.N became almost twice, 1.8 times of that of the time of TTFD administration. It was 1.15 times for R.Y and 1.14 times for Y.Y. Dr. Mori reported before that acid administration increases urinary vitamin C excretion, so

the effect of citric acid is well known.[8] As this study focused on the effect of citric acid administration on urinary vitamin C and oxalic acid excretion, we did not measure urinary citric acid excretion. It is said that vitamin B_1 deficiency increases or decreases citric acid. The administration of citric acid increased urinary vitamin C excretion twice from 3,5mg% before the administration to 6.6mg% for S.N. Vitamin C deficiency seems to be related to the increase of citric acid and pyruvic acid in organism. As universally said, citric acid seems to hinder the utilization of vitamin C in a body as one cause of vitamin C deficiency in anemia.

Table 5 Urinary Vitamin C Excretion at the time of Citric Acid Administration (mg%)

A	B	S.N.	C	S.F.	C	R.Y.	C	Y.Y.	C
7. 7	1	4.1	0.40	2.2	1.00	3.1	0.96	4.2	0.71
	2	1.6		2.2		3.0		3.0	
8	1	3.2	0.83	2.9	0.83	3.4	0.85	3.8	0.86
	2	2.7		2.4		2.9		3.3	
9	1	12.7	0.73	10.1	0.75	11.1	0.72	13.0	0.69
	2	9.0		7.6		8.0		9.0	
10	1	10.6	0.87	10.7	0.72	7.8	0.92	7.7	0.82
	2	9.2		7.2		7.2		6.4	
	D	6 6	0.70	5.6	0.82	5.8	0.68	6.3	0.77

A: Date
B: # of urine collection
C: Proportion
D: Average

Table 6 Urinary Oxalic Acid Excretion at the time of Citric Acid Administration (mg%)

A	B	S.N.	C	S.F.	C	R.Y.	C	Y.Y.	C
7. 7	1	1.5	0.88	9.0	0.90	8.0	0.86	7.5	0.73
	2	4.0		8.1		6.9		5.5	
8	1	4.2	0.92	12.0	0.84	7.9	0.85	6.3	0.90
	2	3.9		10.1		6.8		5.7	
9	1	4.6	0.93	8.8	0.94	8.3	0.86	7.3	0.86
	2	4.3		8.3		6.7		6.3	
10	1	3.9	0.92	9.2	0.85	6.8	1.00	6.9	0.80
	2	3.7		7.9		6.8		6.2	
D		4.1	0.91	9.1	0.91	7.3	0.89	6.5	0.82

A: Date
B: # of urine collection
C: Proportion
D: Average

Table 6 shows the effect of citric acid administration on urinary oxalic acid excretion. The excretion of all the examinees increased by 20, 49, 29 and 20% compared with that before the administration, and showed a considerably higher value than that of the time of TTFD administration except S.N. This seems to result from the fact that citric acid is considered to be an element of oxalic acid production and also to be related to vitamin C utilization.

Thus citric acid has a notable effect on urinary oxalic acid excretion.

Table 7 Urinary Oxalic Acid Excretion at the time of Cane Suger Administration (mg%)

A	B	S.N.	1:2 比 C	S.F.	1:2 比 C	R.Y.	1:2 比 C	Y.Y.	1:2 比 C
7.11	1	3.8	0.82	4.7	0.89	6.5	1.06	6.6	0.71
	2	3.1		4.2		6.9		4.9	
15	1	3.1	0.96	3.9	1.00	5.1	0.90	6.2	0.96
	2	3.0		3.9		4.9		6.0	
16	1	3.6	1.00	4.7	1.00	4.7	0.91	5.8	0.88
	2	3.6		4.7		4.3		5.1	
17	1	3.1	1.20	3.4	0.89	5.1	0.87	5.0	0.64
	2	3.7		3.0		4.0		3.2	
	D 平均	3.5	0.99	4.0	0.94	5.2	0.93	5.3	0.80

Table 8 Urinary Vitamin C Excretion at the time of Cane Suger Administration (mg%)

A	B	S.N.	1:2 比 C	S.F.	1:2 比 C	R.Y.	1:2 比 C	Y.Y.	1:2 比 C
7.14	1	2.0	1.05	2.5	1.08	2.9	0.96	3.5	0.97
	2	2.1		2.7		2.8		3.1	
15	1	3.2	0.90	4.2	1.00	3.9	0.88	4.3	0.97
	2	2.9		4.2		3.9		4.2	
16	1	4.0	0.94	7.9	0.97	6.9	0.98	7.9	0.88
	2	4.7		7.7		6.8		7.0	
17	1	4.8	0.90	7.8	1.01	7.0	0.94	7.7	1.01
	2	4.3		7.9		6.6		7.8	
	D 平均	3.6	0.95	5.3	1.02	5.0	0.94	5.7	0.66

A: Date
B: # of urine collection
C: Proportion
D: Average

(5) Effect of cane suger administration on urinary vitamin C and oxalic acid excretion

Since we used the cane suger solution in oral administ-

ration of citric acid, we studied the effect of this solution. Tables 7 and 8 show the results of the administration of 200 ml water containing 30g cane suger. There was little difference in urinary oxalic acid excretion before and after the administration. Therefore cane suger does not affect urinary vitamin C and oxalic acid excretion.

Summery

The following is the results of the study on effect of the oral administration of TTFD and citric acid on urinary vitamin C and oxalic acid excretion in adult humans.

(1) By the administration of TTFD, the rate of utilization of vitamin C is steadily increased and the amount of urinary oxalic acid excretion also increases.

(2) The citric acid administration lowers the utilization of vitamin C especially in the patients with anemia, and it caused the deficiency of vitamin C, which suggest some disturbances of its utilization.

(3) The influence of citric acid on urinary oxalic acid excretion is considerably strong and citric acid is considered to be an element of oxalic acid production in organism.

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尿中ビタミンCおよび修酸の排泄におよぼす Thiamine tetrahydrofurfuryldisulfide ならびにクエン酸投与の影響

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EFFECT OF ADMINISTRATION OF THIAMINE TETRAHYDRO-FURFURYLDISULFIDE AND CITRIC ACID ON THE URINARY VITAMIN C AND OXALIC ACID EXCRETION

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The present investigation was undertaken to study the effects of TTFD*1) and citric acid on the urinary vitamin C and oxalic acid excretion in adult humans. In the first experiment, 4 adults were ingested with 50mg of TTFD daily for 4 days. In the second experiment, 4 adults were ingested with 5 mg of citric acid for 4 days. By the administsation of TTFD, rate of utilization of vitamin C was steadily increased, and the amount of urinary oxalic acid also increased. By the administration of citric acid, the rate of vitamin C utilization was lowered, especially in the patients of anemia, and it caused the deficiency of vitamin C, which suggests some disturbances of its utilization. The influence of citric acid was considerably strong.

Cの代謝にかんする既往の報告はきわめて多く，またこれに関連した修酸の代謝についても多数の文献がある．

従来修酸は体内におけるC代謝の最終産物と考えられていたが必ずしもそうではなくCはエネルギー源としても利用されるといわれる．血中，尿中の修酸は病態生化学的意義が大きく肝硬変，脚気，化膿性疾患などにおいて増加し，また神経痛，ロイマチスなどの疾患で血中にその蓄積が認められ，腎障害，尿路結石に及ぼす影響も大きい．これらの諸疾患々者にたいしB1とCを併用すると治療効果があがるといわれている．

生体内で糖およびクロロフィルから修酸を生じ，またクエン酸から修酸と酢酸を生成し尿中に排泄される．Cの代謝によって生成した修酸は尿素と結合して$NH_2CONHCOCOOH$の型で存在するが，修酸生成の1因であるクエン酸は糖の中間代謝物の1つであって体内の常成分でKrebs回路の重要化合物である．ビルビン酸からクエン酸を生成する過程においてB1が必要でB1欠乏ではクエン酸は減少する．かく修酸とクエン酸は糖代謝とは深い関係にあり，CとB1との関係も大きい．

さきに著者は経口投与C量と尿中排泄修酸量との間には正の相関があり，また女性生理期における修酸量はその前後より低いことを報告した[3]．上述の如くクエン酸と修酸およびCとB1との関連性に着目し成人にTTFD*1)およびクエン酸を経口投与したばあいの尿中Cおよび修酸の排泄量に及ぼす影響を検討した．

実 験 方 法

(1) 被検者

18才の女子2名，19才の女子1名，36才の男子1名の4名を被検者とした．S.N.はごく軽い貧血症，

*1) TTFD=Thiamine tetrahydrofurfuryldisulfide

S.F.は神経性胃腸炎，R.Y.およびY.Y.は健康であったが後者は茶の多飲者である．

(2) 採尿

午前10時30分と午後0時30分の2回，採尿カップに排尿せしめただちに実験に供した．

(3) 試験期間

昭和42年6月23日から7月17日まで1日間の単位で実施した．

(4) TTFDおよびクエン酸の投与

尿排泄C量は午後2時から3時ごろに増し朝・昼食の影響のあることを考慮しTTFD(50mg)およびクエン酸(クエン酸5gを庶糖30gを含む水200mlに溶かしたもの)の投与は午前9時に行なつた．

(5) 修酸定量法

Bergorman・Ellioの変法[3]により，比色は515mμで日立分光々度計Model 101を使用した．

(6) C定量法

佐藤らのIndophenol法[4]および藤田・照内のPhenylhydrazine法[5]により，比色は530mμで上記分光光度計により行なつた．

実験結果および考察

(1) 尿量とpH

各被検者の投与試験前の尿量平均値はそれぞれ69, 57, 23, 101ml, 庶糖添加時では67, 63, 32, 100ml, TTFD投与時では71, 58, 30, 92ml, クエン酸投与時では56, 62, 34, 102mlであつた．R.Y.では尿がきわめて少なく，Y.Y.では100mlと多く，TTFD, クエン酸および庶糖を投与しても尿量の変動は認められなかつた．pHは5.1～7.2で変動は認められなかつた．

(2) 投与試験開始前尿中のCおよび修酸量

表1にTTFD, クエン酸投与前の各被検者の尿中排泄C量を示す．定量値は試験日1日間のうち前半の2日間はPhenylhydrazine法により，後半2日間はIndophenol法によつたもので以下の実験も同様である．

S.N.の平均C量は3.58mg%であつたのにたいしてS.F.では5.60mg%と最も多くS.N.の1.7倍に達した．R.Y., Y.Y.では5.15～5.53mg%を示した．Y.Y.は茶の多飲者であり比較的多量のC排泄を認めたものであろう．一般に貧血のばあい骨髄の赤血球生成機能に重要な役割を果すというCが欠乏するといわれ[6]また慢性C欠乏症にあつて低鉄血症，貧血がおこり，このばあい鉄剤のみでは回復は著しくなく，Cの併用が有効といわれている[7]．S.N.のばあいこのことを暗示するものであろう．

表2には投与試験前の尿中修酸を示しS.N.では他の被検者に比べ3.3mg%とかなり低値でY.Yでは5.15mg%と高かつた．尿中C量と修酸量は正の相関が認められる．

(3) TTFD投与の尿中Cおよび修酸に及ぼす影響

表3にTTFD 50mg投与時の尿中C排泄量を示す．被検者4名のC量は3.5, 5.5, 5.1および5.6mg%で投与前排泄量に比べS.N., S.F., R.Y.の3名では減少が認められた．すなわちTTFD添加により2%程度減少

表1 尿 中 C 量 (mg%)

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
6. 23	1 *	2.59	0.84	3.12	0.93	2.73	1.03	3.47	1.01
	2 **	2.19		2.90		2.81		3.51	
24	1	3.00	1.00	4.29	0.93	3.81	1.05	4.51	0.93
	2	3.00		4.01		1.00		4.22	
25	1	4.66	0.84	7.70	0.99	7.15	0.88	7.64	0.82
	2	4.00		7.65		6.27		6.30	
26	1	4.79	0.91	7.57	1.00	6.98	1.03	7.78	0.87
	2	4.40		7.60		7.22		6.80	
	平均	**3.58**	**0.89**	**5.60**	**0.96**	**5.15**	**0.99**	**5.51**	**0.91**

* 10時30分採尿　　** 12時30分採尿

表2　尿中修酸量 (mg%)

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
6. 23	1	2.0	1.30	4.7	0.85	4.2	0.95	4.2	0.93
	2	2.6		4.0		4.0		3.9	
24	1	3.4	1.25	5.1	0.94	4.8	0.94	6.1	0.95
	2	4.4		4.8		4.5		5.8	
25	1	3.0	1.30	4.9	0.82	7.4	0.93	7.2	0.70
	2	3.9		4.1		6.9		5.0	
26	1	3.8	0.93	5.3	0.94	5.1	0.90	4.9	0.82
	2	3.6		5.0		4.6		4.1	
	平均	**3.3**	**1.20**	**4.7**	**0.89**	**5.2**	**0.93**	**5.15**	**0.85**

表3　TTFD添加時の尿中C量 (mg%)

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
6. 30	1	2.1	0.52	5.4	0.70	3.9	0.80	4.7	0.80
	2	1.1		3.6		3.0		3.7	
7. 1	1	3.0	0.90	4.3	0.81	3.8	1.00	5.0	1.00
	2	2.7		3.5		3.1		5.0	
2	1	4.9	0.90	7.9	0.76	7.4	0.80	7.7	0.80
	2	4.4		6.0		6.2		5.9	
3	1	5.1	0.96	7.6	0.80	7.0	0.72	7.8	0.72
	2	1.9		5.9		6.4		5.6	
	平均	**3.5**	**0.82**	**5.5**	**0.77**	**5.1**	**0.83**	**5.6**	**0.83**

表4　TTFD添加時の尿中修酸量 (mg%)

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
6. 30	1	4.2	1.10	5.3	0.73	4.9	0.96	5.5	0.62
	2	4.6		3.9		4.7		3.4	
7. 1	1	4.1	1.10	5.9	0.97	5.2	0.96	7.7	0.90
	2	1.9		5.7		5.1		7.0	
2	1	5.3	1.30	5.0	1.02	5.5	1.00	5.6	0.71
	2	1.0		5.1		5.5		4.0	
3	1	4.2	0.93	5.5	0.80	5.5	0.90	6.0	0.90
	2	3.9		4.7		4.9		5.4	
	平均	**4.2**	**1.08**	**5.1**	**0.89**	**5.1**	**0.95**	**5.6**	**0.78**

したことはTTFD添加によつて生体内代謝が促進され，これに応じて酸化還元反応が進みCの使用量が増したものと推測される．

なお本研究着手前にB1負荷試験を行なつたところB1服用前2時間尿のB1量はそれぞれ60，70，76および110γとなり，B1 5mgの投与による2時間尿中B1排泄増加率は7，9，12および11%であつた．この結果からすればS.N.はすでにB1不足の状態にあつたもののようで，S.F.ではB1摂取量はやや少なく，他の2名は正常のB1摂取状態にあつたことがわかる．したがつてS.N.，S.F.2名のC排泄量の減少は代謝の促進の結果によるものと解釈できる．

表4によればTTFD投与によつて尿排泄修酸量も増加することがわかる．S.N.，S.F.およびY.Y.で24，9および8%の増加を示したがR.Y.では2%の減少が認められた．このことはいちおうTTFDの投与は修酸の排泄を促進するものと考えられる．

4) クエン酸投与の尿中Cおよび修酸に及ぼす影響

表5に示すように各被検者の尿中C量は6.6，5.6，5.8および6.3mg%でTTFD投与時に比べるとつた．とくにS.N.では2倍程度となり投与時に比べ1.8倍と多く，R.Y.では1.15倍，Y.Y.では1.14倍であつた．酸の投与によつて尿排泄C量の増加することはすでに森の報文[9]があり，クエン酸の影響が大きいことが知られている．本研究では尿中排泄C量と修酸量に及ぼす影響に重点をおいたので尿中クエン酸の測定は行なわなかつた．クエン酸はB1不足で減少するとも[10]，あるいは逆に増加するともいわれている．とくに肝疾患，貧血で増量するという．クエン酸の投与でS.N.では無投与時排泄C量3.5mg%であつたのが6.6mg%と2倍

表5 クエン酸添加時の尿中C量 (mg%)

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
7. 7	1	4.1	0.40	2.2	1.00	3.1	0.96	4.2	0.71
	2	1.6		2.2		3.0		3.0	
8	1	3.2	0.83	2.9	0.83	3.4	0.85	3.8	0.86
	2	2.7		2.4		2.9		3.3	
9	1	12.7	0.73	10.1	0.75	11.1	0.72	13.0	0.69
	2	9.0		7.6		8.0		9.0	
10	1	10.6	0.87	10.7	0.72	7.8	0.92	7.7	0.83
	2	9.2		7.2		7.2		6.4	
	平均	**6.6**	**0.70**	**5.6**	**0.82**	**5.8**	**0.68**	**6.3**	**0.77**

表6 クエン酸添加時の尿中修酸量 (mg%)

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
7. 7	1	4.5	0.88	9.0	0.90	8.0	0.86	7.5	0.73
	2	4.0		8.1		6.9		5.5	
8	1	4.2	0.92	12.0	0.84	7.9	0.85	6.3	0.90
	2	3.9		10.1		6.8		5.7	
9	1	4.6	0.93	8.8	0.94	8.3	0.86	7.3	0.86
	2	4.3		8.3		6.7		6.3	
10	1	3.9	0.92	9.2	0.85	6.8	1.00	6.9	0.80
	2	3.7		7.9		6.8		6.2	
	平均	**4.1**	**0.91**	**9.1**	**0.91**	**7.3**	**0.89**	**6.5**	**0.82**

の増加を示し，C欠乏は組織中クエン酸の増量，ヒルビン酸の増量[11][12]と関係があるようで，一般にいわれるように貧血症におけるC欠乏の1因としてクエン酸は生体内におけるCの利用妨害作用のあることが推定される．

表6に修酸排泄に及ぼすクエン酸投与の影響を示す．各被検者ともに無投与時に比べ0，19，29および20%と排泄率は増しTTFD投与時に比べるとS.N.を除きかなり高い値となった．前述のように生体内における修酸の生成源としてクエン酸も考えられ，またC代謝とも関係することからこのような結果が得られたものであろう．このクエン酸の尿排泄修酸に及ぼす影響はかなり大きいことが注目される．

(5) 庶糖投与の尿中Cおよび修酸に及ぼす影響

クエン酸を経口投与するさいに庶糖水溶液を用いたのでこのものの影響について検討すべく30gの庶糖を200mlの水に溶かして投与した結果を表7，8に示す．すなわち試験前と投与後の修酸排泄量とはほとんど差がなかったから庶糖はCおよび修酸の排泄量にほとんど影響がないことを知った．

結　　論

成人にTTFDおよびクエン酸を経口投与し尿中Cおよび修酸排泄量に及ぼす影響を検討して，つぎの結果を得た．

(1)　TTFD投与により生体内Cの利用率は高まり修酸の排泄量も増す．

(2)　クエン酸投与によりCの利用率は低下するもののようで，とくに貧血疾患者にあってはC欠乏の1因としてこの利用妨害のあることが推察される．

表7　庶糖添加時の尿中修酸量（mg%）

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
7. 14	1	3.8	0.82	4.7	0.89	6.5	1.06	6.6	0.74
	2	3.1		4.2		6.9		4.9	
15	1	3.1	0.96	3.9	1.00	5.1	0.90	6.2	0.96
	2	3.0		3.9		4.9		6.0	
16	1	3.6	1.00	4.7	1.00	4.7	0.91	5.8	0.88
	2	3.6		4.7		4.3		5.1	
17	1	3.1	1.20	3.4	0.89	5.1	0.87	5.0	0.64
	2	3.7		3.0		4.0		3.2	
	平均	**3.5**	**0.99**	**4.0**	**0.94**	**5.2**	**0.93**	**5.3**	**0.80**

表8　庶糖添加時の尿中排泄C量（mg%）

試験日	採尿回	S.N.	1:2比	S.F.	1:2比	R.Y.	1:2比	Y.Y.	1:2比
7. 14	1	2.0	1.05	2.5	1.08	2.9	0.96	3.5	0.97
	2	2.1		2.7		2.8		3.4	
15	1	3.2	0.90	1.2	1.00	3.9	0.88	4.3	0.97
	2	2.9		1.2		3.0		4.2	
16	1	4.0	0.94	7.9	0.97	6.9	0.98	7.9	0.88
	2	4.7		7.7		6.8		7.0	
17	1	4.8	0.90	7.8	1.01	7.0	0.94	7.7	1.01
	2	4.3		7.9		6.6		7.8	
	平均	**3.6**	**0.95**	**5.3**	**1.02**	**5.0**	**0.94**	**5.7**	**0.66**

3) クエン酸の尿排泄修酸量に及ぼす影響はあまり大きく，生体内修酸生成源としてクエン酸もその1つと考えられる．

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人におけるビタミンCの尿中排泄にたいするフラボノイドの影響

8人ずつの2群の患者に毎日500mgのAsAを投与し1群には400mgのFlavonoidを併用し19日間にわたり尿中AsA排泄量を比較した．3日の予備期間および11日間の投与期ともに両群に差が認められなかったが最後の5日間ではFlavonoid投与群の方がAsA排泄量が著明に高かった．細胞の呼吸機作にたいしFlavonoidがAsAと関連をもちAsAを節約するものではないかと思われる．(Demole, M., Horn, R., von der Mühl, M. : Intern. Z. Vitaminforsch. **37**, 496, 1967)

(高田)

ネズミにおけるビタミンAの吸収に及ぼす胆汁の影響

長鎖脂肪酸は腸管吸収にあたって腸粘膜中でトリグリセリドとなりリンパ径路を通るが胆汁が欠除したばあいには門脈とリンパとの分配に変化を来し門脈径路を通るものが多くなると考えられている．この考え方をAのばあいについて検討してみた．胆管ろうをつくったネズミの1群にさらに胆嚢ろうを作製したのちA-^{14}Cを投与しリンパ液，肝および消化管内容物中の脂溶性^{14}Cを測定した．その両群ともに回収率が低かったがことに胆汁ろう動物にいちじるしく低率であった．これは腸内バクテリアのために水溶性^{14}C化合物となったためと思われる．胆嚢ろうを有しない対照動物では吸収Aの90%はリンパ液に検出されたのに胆嚢ろう動物では52%にすぎず，また^{14}Cのリンパ：肝の比は前者の18にたいして後者では1.5にすぎず，対照における吸収Aは主としてリンパ径路で運ばれるのに反し胆汁を欠くばあいにはこの径路が障害され肝中に多く検出されることを知った．(Gagnon, M., Dawson, A.M. : Proc. Soc. Exp. Biol. Med. **127**, 99, 1968)

(高田)

葉酸投与によるだ液中の葉酸活性の変化

だ液中のFA活性はFA投与によって変化することが知られ絶食時の耳下腺だ液中のFAはほとんど0である．著者らは耳下腺だ液を顎下腺＋舌下腺だ液に分けて採取し*L. casei*によるFA活性を10例について測定した．絶食時には耳下腺だ液ではすべて0であったが混合だ液では平均0.25mμg/mlのFAを含有していた．これに10mgのFAを経口投与し経時的にFA活性を測定した結果，1時間後から急に上昇し4時間後には最高となり6時間後もなお絶食時より高値を保っていた．最高値は混合だ液では1 mμg/ml，耳下腺だ液では0.3 mμg/mlであった．混合だ液の方がFA活性の高いのはバクテリアや脱落表皮などを夾雑するためと思われる．(Makilä, E., Kirveskari, P. : Intern. Z. Vitaminforsch. **37**, 487, 1967)

(高田)